# SELECTIVE TOXICITY

ADRIEN ALBERT

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METHUEN'S MONOGRAPHS ON BIOCHEMICAL SUBJECTS

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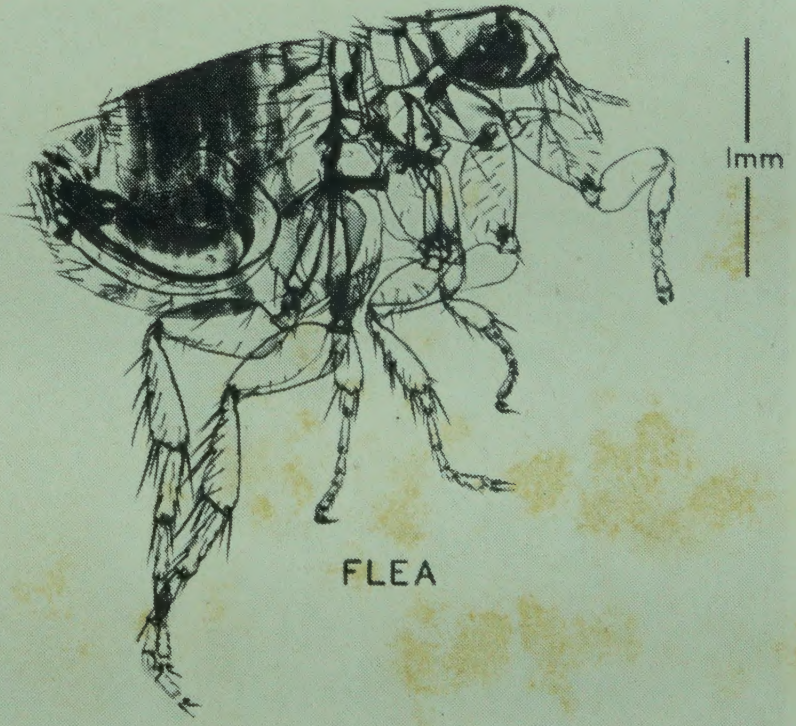
METHUEN'S MONOGRAPHS ON BIOCHEMICAL SUBJECTS
*Edited by*
R. A. PETERS, Whitley Professor of Biochemistry in the University of Oxford, and F. G. YOUNG, Sir W. Dunn Professor of Biochemistry in the University of Cambridge

# SELECTIVE TOXICITY

# CHART OF SIZES


DOG

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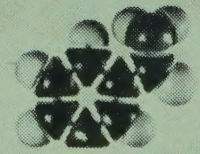


MOLECULE
(NICOTINAMIDE)

This chart is to help one remember the relative sizes of Molecules, Microbes, Insects and Mammals. In each case a fairly small example has been chosen (*e.g.* Dog and not Whale). Each object is drawn with a magnification 1000 times greater than the object preceding it.

# ELECTIVE TOXICITY

with special reference to

# CHEMOTHERAPY


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# *PREFACE*

HIS is a book about selectively toxic agents, that is to say, bstances which injure some kinds of cells and not others, en when the two kinds are growing close together. Hence e book is concerned with drugs and weed-killers and ecticides. It is not very much concerned with *what* these bstances accomplish, but with the physical and chemical eans by which they accomplish it.

The book grew out of some postgraduate lectures which ofessor F. G. Young encouraged me to give at University llege, London, in 1948 and 1949. The lectures were tended by research workers and senior students in bio-emistry, chemistry, medicine, veterinary science and riculture, and these very different categories of listeners l seemed to find something interesting in them. Hence is to similar categories of readers that this book is ldressed. I hope that they will find it reasonably self-planatory, even on a first reading, and that they will ad it again, later, making use of the footnotes and the ferences which have been inserted to aid more serious udy. I also hope that the book may come to be used as text for advanced classes and for those discussion groups which biologists and chemists meet to build a bridge tween their diverse disciplines. Above all, this book is tended to help people who wish to get away from mere aper resemblances' between the formulae of molecules and base their reasoning on something more fundamental.

I am only too well aware of the complexity of my subject. owever, complexity is inherent in all biological subjects d has not prevented the discovery of valuable basic inciples. Selective toxicity, which has for so long been branch of technology rather than of science, is ripe for e discovery of such underlying principles.

The most valuable approach to problems of how biologically active substances work is the method of the *limiting factor*. The first step in this method is to investigate and record the various physical, chemical and biochemical properties of active substances. Parenthetically, it may be pointed out that too little insight and imagination is usually brought to this elementary procedure so that the next step cannot be made effective for want of data that could be of *biological* relevance. The second step consists of supposing, in turn, that each of these properties plays a leading part in the biological action of the substance. To this end, each property, in its turn, is made the limiting factor in a series of experiments which are designed in the following way. Using only one substance, the conditions of test are repeatedly changed in a way that makes the limiting property appear in different degrees of intensity. The experimenter then observes whether the biological activity varies in proportion to the intensity of this property. Such experiments are followed or preceded by others in which the conditions of test are kept constant, but instead of confining the tests to a single substance, various substances are used, substances which differ from the original material in every way except as to the property being examined. Several examples of the method of the limiting factor are described here, e.g. in Chapter IV.

In writing this book I have thought it important to give most space to those agents whose mode of action is best known, regardless of whether they are the toxicants of greatest economic importance. It is likely that the number of kinds of ways in which selectively toxic substances can act is limited. Hence, the mode of action of even the least spectacular drug, if really well understood, is likely to help in deciphering the nature of other selectively toxic agents.

Because of the limitations of time, some interesting divisions of the subject did not find a place in the original lectures, and limitations of space has prevented them from appearing here. For instance, it would be appropriate to

something about the influence of oxidation-reduction tentials and much more about surface-chemistry. reochemical effects, too, deserve more space than they given; for example, it would have been interesting to cuss why the *d*- and *l*-modifications of a drug sometimes ve identical action (as in cocaine and in the antimalarial, loroquin), whereas in other cases only one of the optical merides has the desired type of activity. However, I pe that the selection of topics included here will be found ceptable and that it will evoke further reading along rallel lines. *The Basis of Chemotherapy*, by T. and E. ork (1948) and *Cell Physiology and Pharmacology* by F. Danielli (1950) can be recommended for this purpose.

In conclusion I should like to thank Professor F. G. oung, F.R.S., for having given me so much encouragement in writing this book. I am grateful to many past lleagues for stimulating discussions which have borne uit here, in particular to B. Breyer, J. E. Falk, R. J. oldacre, F. G. Lennox, R. N. Robertson, W. P. Rogers, D. Rubbo, E. Singer, and J. M. Vincent. I thank rofessor Ernest Baldwin and the Cambridge University ress for permission to reproduce Figure 2 (Chapter I) om "Comparative Bio-chemistry". I wish to thank Miss izabeth Thomas for preparing the manuscript for the ess and for her help in proof-reading.

# CONTENTS

## *PLATES*

## *CHAPTER ONE*

) WHAT IS SELECTIVE TOXICITY?

) THE PRACTICAL ACCOMPLISHMENTS OF SELECTIVE TOXICITY

) THE SCIENTIFIC BASIS FOR SELECTIVE TOXICITY, IN TERMS OF BIOLOGY AND THE PHYSICAL SCIENCES

### *a) What is selective toxicity?*

At the beginning of the present century, it was often ebated whether it would become practicable to poison ving cells selectively, i.e. without injuring other cells with hich they were associated. In other words, it was doubted hether any substantial practice of selective toxicity could ver be realized. Yet to-day it is an accomplished fact.

The main theme of this book is selective toxicity in its cientific aspects rather than in its applications. Yet, many f these applications will be discussed because they provide amiliar examples on which to hang the scientific discussion. However, the principal emphasis is laid upon the scientific rinciples of selective toxicity because of their value in nterpreting biological activity and because it is expected hat a study of these principles will eventually lead to mportant developments which might otherwise be missed.

In general terms, the story of selective toxicity is the story f some of the most difficult economic problems which han has met and overcome in his struggle to retain mastery ver his environment. Let us start with a definition of elective toxicity as the practice of injuring one species of ving matter without harming another species with which he first is in intimate contact. We may think of the species hich is to be injured as the *uneconomic species* and the pecies which is to be preserved as the *economic species*.

Often these are related to one another as parasite and hos but in other cases they are purely commensal.

In agriculture, the need for selective toxicity has lon been felt. Man needs to grow crops of plants in order t obtain food and clothing. The seeds of these plants, th soil around the plants and the plants themselves are com monly infected with all sorts of fungi, weeds and insec pests which threaten the economic success of the crop Obviously, it has been an achievement of prime importanc to have found those selectively toxic agents which ar now used in agriculture to remove many of the uneconom species without injury to the economic ones.

In the animal industries a similar situation is recog nizable. Man's economic animals are afflicted by all kind of parasites, external and internal. As in agriculture, th continuance, not to mention expansion, of the anima industries depends on the constant discovery of improvec selectively toxic agents.

Man, himself, is no less subject to endo-parasites than his economic animals. Chemotherapy is the name given tc that branch of selective toxicity which is concerned with the removal of parasites from man and his tended animals. So far, more has been discovered of the scientific principles underlying the practice of *chemotherapy* than of any other branch of selective toxicity. For this reason we shall be drawing very freely on the literature of chemotherapy with the expectation that what we discover there will illuminate the more obscure corners of our subject.

Thus far, we have confined ourselves to a definition in which the economic and uneconomic cells constitute, respectively, two distinct species. But the more complex case can now be considered where the uneconomic cells are part of the organism of the economic species. For example, the uneconomic cells may be a malignant growth in an otherwise healthy host; it may be a gland that has hypertrophied and upset the balance of metabolism of an otherwise healthy body; it may simply be some part of the

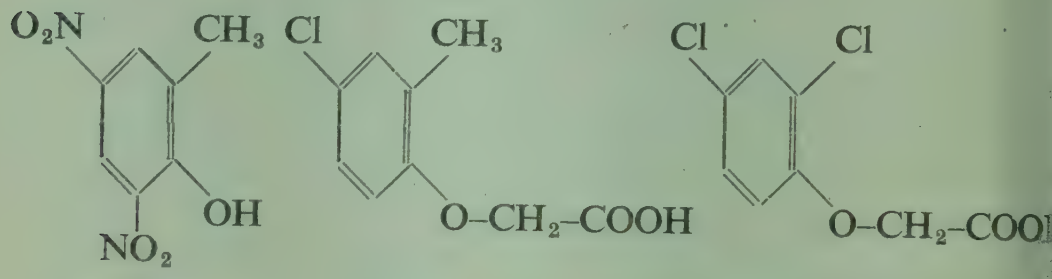
vous system which has become overactive and has rupted the harmony in which bodily functions normally rk. All of these morbid conditions call for the discovery l investigation of selectively toxic agents: the branch of ctive toxicity which is concerned with this work is own as *pharmacology*. In general, the scientific study of rmacology seems to be proving more difficult than that chemotherapy. In pharmacology, selectively toxic agents often required to have a temporary rather than a perma- nt action. For instance, the abolition of the ability to l pain, whether in a circumscribed area or quite generally, one of the great triumphs of pharmacological practice; t it would not be counted a triumph if anaesthesia were persist throughout the remainder of the patient's life.

*The practical accomplishments of selective toxicity*

We now have some idea of the scope of our subject; let review briefly some of the major practical accomplish- nts. We shall deal firstly with the achievements of ective toxicity in the realm of agriculture, and I have ought it worth while going into this branch of the subject some detail because so many of the applications to man and veterinary medicine will receive mention in the urse of the remaining lectures.

It is fifty years since Bonnet showed, in France, that low charlock could be killed in a field of oats, without ury to the crop, by spraying a solution of copper sulphate er the whole field. In 1911 another Frenchman, Rabaté, owed that a solution of sulphuric acid could be safely ed on crops to destroy the weeds. It was not until 1932 t systematic trials of Rabaté's method were made in gland. This was the year in which two more Frenchmen, uffaut and Pastac, discovered the selective weed-killing operties of dinitro-*o*-cresol (I), a substance which had en known as a chemical curiosity since 1866. By the ddle 1930's, a good deal of the prejudice against the idea selectively toxic weed-killers had been overcome. The

agricultural industry was thereafter receptive to fresh selec tive weed-killers such as the phenoxyacetic acids whicl Slade, Templeman and Sexton found in 1942 while investi gating the spraying of cereal crops with substances know to promote the growth of roots in other species.




(I) (II) (III)

At the present time, the most versatile of the weed-killers are dinitro-*o*-cresol (I), 'Methoxone' (II, 2-methyl-4-chlorophenoxyacetic acid) and '2,4-D' (III, 2 : 4-dichloro-phenoxyacetic acid). Tests have been carried out on thirty annual weeds that normally impoverish cereal crops. The results clearly show that by choosing the right compound and applying it at the right time, almost all the weeds can be killed. Large-scale practice has substantiated this conclusion. A certain amount of specificity is shown; for example, corn buttercup and shepherd's needle are destroyed best by 'Methoxone', whereas dinitro-*o*-cresol is the better agent against corn marigold and poppies. A few ounces of these organic substances suffice for an acre; this high degree of activity suggests that their mode of action is a very specific one.

Unfortunately the biochemistry of this mode of action is unknown, although in general terms it is a matter of the weeds responding to the chemicals by a burst of metabolic stimulation and accelerated growth which rapidly exhausts all reserves. It is not known why cereals are relatively unaffected. Experiments have been conducted where cereals have been made to absorb as much of the phenoxy-acetic acid as the weeds do (normally they absorb somewhat

), but the cereals have remained unharmed (Wood, lfe and Irving, 1947). On the whole, dicotyledons are ed and monocotyledons survive, but this is not the sole se of the difference because (I), (II) and (III) are ruinous onion plants which are, of course, monocotyledons.

The introduction of organic weed-killers has been a mendous economic gain. They are non-poisonous to n and animals; non-corrosive to machinery. They have ected an overall increase of 20 per cent in the grain crops this country and, in the case of barley, increased yields up to 90 per cent have been recorded. These substances ve been just as successfully used on crops of flax and of sses, to say nothing of their domestic use in removing eds from lawns.

Unfortunately there is, as yet, no practicable method r chemically weeding vegetable crops. Some interesting periments have been carried out with *iso*propyl phenyl-rbamate which kills grasses but spares many dicotyle-nous plants.

Before leaving the subject of selective toxicity in cereal ops, let us dwell a little longer on sulphuric acid, because shall frequently have occasion to refer to this example. en since the advent of the organic weed-killers, a 20 per nt solution of sulphuric acid is still considered the best ent for removing chickweed from a wheat crop (Black-an, 1946). This use of sulphuric acid may well become ssic because it demonstrates how a selectively toxic effect n be obtained by making use of suitable distribution enomena. Sulphuric acid is just as injurious to the toplasm of wheat as it is to that of chickweed. The portant difference is that the chickweed is rough and the eat smooth, so that the sulphuric acid is accumulated the chickweed but shed from the wheat.*

* To particularize: 'The leaves of cereal crops are waxy and also upright; growing tissues are basal and protected by leaf-sheaths. Thus the wax vents penetration, the droplets run off the leaves and the meristems do not ne in contact with the spray. On the other hand, most dicotyledonous ds have flat waxless leaves, which catch the spray, while the growing point xposed and vulnerable, since it is the apex of the shoot.' (Blackman, 1947).

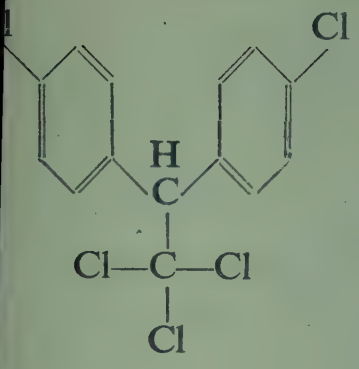
We shall frequently come across cases, in the following chapters, where distribution effects play an important part in determining selective toxicity, but we shall seldom find distribution acting in such naked isolation as in this example.

Streptomycin, possibly, provides the nearest example. This antibiotic interferes with the reaction between pyruvic and oxalacetic acids in the Krebs' cycle, a reaction which seems to be essential for all forms of life. However, in the animal this reaction is localized in the mitochondria, which are cellular granules having a permeability barrier. In the bacterial cell, the reaction is not protected in this way and thus bacteria are injured by concentrations of streptomycin which are harmless to the animal cell (Umbreit and Tonhazy, 1949).

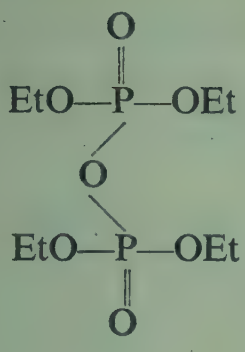
Although the chemical removal of weeds from crops is of fairly recent origin, the removal of insect pests dates back farther. Inorganic substances such as lead acetate, sulphur and copper sulphate were principally used and later such vegetable products as tobacco dust and powdered pyrethrum were introduced. A striking advance was made by the introduction of DDT (IV) during the Second World War. It is hardly necessary to go into details of the immense economic value of DDT in the control of agricultural insect pests. This subject has been dealt with very fully by West and Campbell (1946). For example, DDT has proved effective against Colorado beetle (in potatoes), apple-blossom weevil and the tomato moth. On the other hand, it is not very effective against aphids, thrips, red spider and slugs.

Tetraethyl pyrophosphate (V) and parathion ('Thiophos', VI), which were discovered by Schrader in Germany during the Second World War are now being used to control species of insect pest (e.g. red spider) which DDT does not injure. All three new substances are reasonably non-toxic to man, and are used rather more freely than nicotine or lead arsenate.*

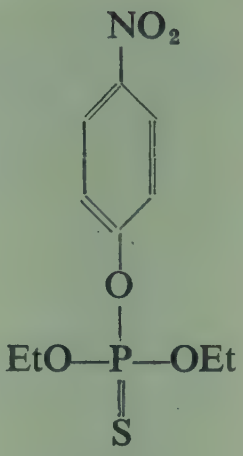
* Tetraethyl pyrophosphate is only non-toxic to man by virtue of the fact that it becomes hydrolysed within a day or two after application to plants.



(IV)

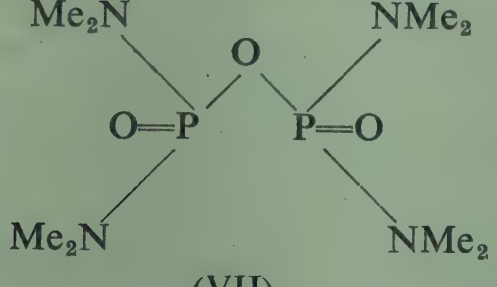


(V)



(VI)

There are yet other sections of agriculture in which lectively toxic agents have been put to work. Seeds, articularly grain seeds, are commonly dusted with phenyl ercuric nitrate during storage to prevent infection by ngi, which hitherto caused great losses on storage. This eatment does not affect germination of the seeds. Then ere is chemo-prophylaxis against insects. It is now pos-ble to impregnate the soil with substances so designed at the plant will take them up during growth. These bstances are non-toxic to plants but highly toxic to sects. A typical example is di(β-fluoroethoxy)methane. his substance accumulates in the sap and kills the insects soon as they bite the plant. Unfortunately, such sub-ances are (so far) all poisonous to mammals so that they n be used only by seed-raisers and flower growers. owever, *bis*(*bis*dimethylaminophosphonous)-anhydride II) is considered safe for use in peas, hops, brussels routs and sugar-beet under controlled conditions.



(VII)

In veterinary practice there have been many economically valuable applications of selectively toxic agents in spite of the fact that many of the remedies devised for human illness cannot be used in veterinary medicine because of their high cost.

Flies and other biting pests, which so seriously reduce the condition of stock, are being controlled with derris and with DDT. Bovine mastitis, which greatly reduces the yield of milk, is now cured with sulphanilamide. Carbon tetrachloride and phenothiazine are widely used for controlling worm infections in sheep. Coccidiosis, and other diseases of poultry, readily yield to chemotherapy. Trypanosomiasis in cattle, the prevalence of which has prevented the development of immense areas of fertile land in Africa, seems likely to be lessened by the recently discovered drug, 'Antrycide'.

When we turn to the hygienic and medical welfare of human beings, we must note the introduction of improved insect-repellants such as dimethyl phthalate against mosquitoes,* dibutyl phthalate against the mites that carry scrub typhus, and benzyl benzoate against scabies. A history-making event was the use of DDT to prevent an outbreak of typhus from becoming an epidemic in Naples, in January 1944. More than a million civilians were dusted with DDT, thus killing the lice which are the vectors of this dreaded disease. Conditions at that time were such that, had the epidemic developed, the death-roll in Europe would probably have far exceeded the total casualties of the war. DDT has also proved valuable for suppressing the breeding of mosquito larvae, thus exerting a powerful brake on the incidence of malaria. The action against household pests is also notable.

Chemotherapy has made great contributions to human medicine since Ehrlich placed it on its scientific feet at the beginning of this century. Organic arsenicals can cure

* For a discussion of the connexion between repellancy to mosquitoes and physical and chemical properties, see Christophers (1947).

yphilis, formerly one of the most persistent of diseases, in few months. 'Atebrin' has revolutionized the prophylaxis nd treatment of malaria and 'Paludrine' seems likely to nake a further contribution to this. Suramin (Bayer 205) as shown a remarkable prophylactic action in human leeping sickness, a single dose giving protection for three nonths. The sulphonamides and penicillin have com-pletely revolutionized the treatment of streptococcal sep-icaemia, pneumonia and gonorrhoea. Carbon tetrachloride s used every year to treat millions of people who have allen victims to hookworm. Streptomycin has been found valuable in the treatment of certain types of tubercular nfection, and chloromycetin is giving excellent results in yphus.

Tremendous as this saving in life and suffering have been, here is great scope for further efforts. Chemotherapy, for nstance, can do so little for hydatid disease or for infections, such as influenza and infantile paralysis, that are caused by viruses.

Pharmacological developments have also brought a re-markable measure of control over the working of the human body. Patients can be relieved of pain of all types and degrees of severity, put to sleep and awakened, prevented from having convulsions and caused to have them for their therapeutic value. All of these things can be done with simple synthetic chemicals which may be thought of as selectively toxic agents because the patient s unharmed. Similarly, the patient's temperature can be raised or lowered, his sympathetic or his parasympathetic nervous systems can be selectively stimulated or depressed, his basal metabolic rate raised or lowered and the clotting power of his blood can be made greater or less. Moreover, deficiency or hyperactivity in the action of muscles (includ-ng the heart) have come within some measure of control, and so have the activities of a few of the endocrine glands. Excessive secretion of histamine, the cause of so many dis-ressing symptoms, can now be counteracted. The control

of prostatic cancer and, to a lesser extent, of leukaemia are two valuable contributions to the selective toxicity of cancer.

We may conclude our necessarily incomplete review of the present achievements of selective toxicity with the sobering thought that much more remains to be done than has yet been accomplished. In all these various fields of endeavour, man has but stirred the surface. Nevertheless, the successes obtained are so striking that we have the right to expect that the many outstanding problems will be successfully solved. It is reasonable to believe that they will be solved significantly sooner if we constantly keep before our eyes, and store in our minds, the many connexions between constitution and activity which have already been reliably established.

### (*c*) *The scientific basis for selective toxicity in terms of biology and the physical sciences*

In view of these many instances where the principle of selective toxicity has been successfully harnessed for the welfare of man, we may well ask on what scientific basis does this principle rest. In a few cases it is simply a matter of *accumulation*, as in the above instance of sulphuric acid which spared the wheat and spoiled the weed. Accumulation implies some or all of the following: (*a*) efficient transport to the outside of the cell; (*b*) a favourable permeability mechanism; and (*c*) a satisfactory storage mechanism.

Yet, in many instances, accumulation plays a comparatively minor role and several instances are known where a profound physiological effect is obtainable in spite of a poor distribution. As an example of the latter we may consider the powerful oestrogenic drug 'Fenocyclin'* which, after subcutaneous injection, is concentrated 7 and 2·5 times as strongly in the intestines and liver respectively as it is in the uterus, although it acts only on the last-named (Miescher, 1948). Many other examples lend confirmation to the

* Methyl*bis*dehydro-doisynolic acid.

concept that a strong but localized biological activity often depends on a *specific type of reaction*, a reaction which can not take place to anything like the same extent anywhere but in the affected cells.

The moment we adopt this approach we come up against a most formidable mental difficulty. How is it possible for toxic agents to act selectively on different types of cell when the major biochemical discoveries of the last two decades have indicated that there is a *common ground-plan for all living matter*, whether animal, plant or microbiological?

This objection must be taken quite seriously and we might begin by listing the instances on which it is based. Let us group these under the following four headings.

1. *Genetics*. The laws of genetics apply almost universally. Hence a practically universal biochemistry of mitosis can be inferred. Support for this concept comes from toxic agents such as colchicine which arrests the process of mitosis at one particular stage in all the species examined.

2. *Carbohydrate metabolism*. The Meyerhof sequence of glycolysis, shown on p. 12, has been found to apply almost universally. This sequence has been demonstrated in representative bacteria, protozoa, nematode worms, insects, echinoderms and vertebrates, as well as in yeast and the higher plants. There is no essential difference between the glycolysis of such a lowly form of life as yeast and some of the most highly organized tissues (e.g. human muscle and liver). This has been shown conclusively by the use of inhibitors and the actual isolation of enzymes and intermediates.

This scheme of glycolysis requires repeated phosphorylations and these are just as universally carried out by one particular chemical substance, adenosine triphosphate. This substance seems to be irreplaceable for transferring energy in large increments between the various parts of the metabolic cycle, balancing anabolism against catabolism.

3. *Hormones and vitamins*. It is well known that vertebrate hormones influence invertebrates and *vice versa*.

Substances which show the pharmacological action of adrenaline on the frog's heart and rabbit's ear have been extracted from protozoa, annelid worms, molluscs and arthropods (cf. Wense, 1939). Conversely, true adrenaline causes increased muscle-tone in annelida, molluscs and

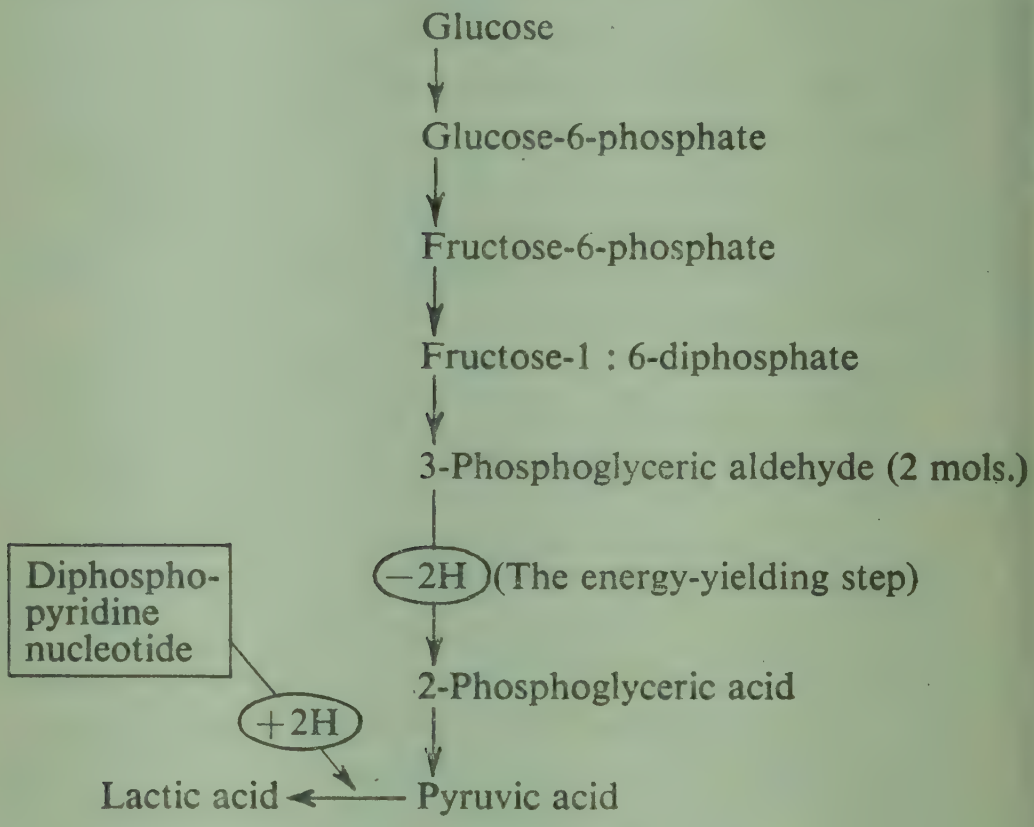



Fig. 1—Glycolysis.

arthropods. Similarly, substances having sex-hormone (oestrogenic) action on vertebrates (mouse-test) have been extracted from protozoa, cœlenterates, annelid worms, molluscs and echinoderms (Steidle, 1930). In general, hormones are not at all specific where species are concerned.

Certain substances, notably thiamine, riboflavine and nicotinic acid, which are members of the B group of vitamins, appear to be essential constituents of all living cells.

4. *Metabolism of foreign substances.* Only eleven different methods are known by which vertebrates destroy

reign substances that are administered to them. These e the so-called detoxication mechanisms, e.g. oxidation, duction, acetylation, sulphation, methylation, coupling th glucuronic acid or cysteine. Nearly all of these echanisms were known in the last century; fifty years of tensive research has uncovered only one more (cf. Villiams, 1947). No such wealth of data has been accu-ulated for invertebrates, but a substantially similar tuation seems to exist there also.

Yet, remarkable as these similarities are, the very fact hat one species behaves differently, and looks different, rom another species encourages us to believe that there nust actually be marked biochemical differences between he various species. For the same reason, there must be narked differences in the biochemistry of various tissues vithin any one species. In proportion as we find such vidence of biochemical differentiation, the biochemical asis of selective toxicity becomes perfectly clear. I have rouped some of the more important pieces of evidence nder seven headings. Most of the instances have been ulled from studies of catabolism because this is the most horoughly investigated branch of metabolism. However, t is likely that catabolic processes, in various species, esemble one another much more closely than anabolic nd reproductive ones where the end-products are so palpably individual and differentiated.

1. *Genetics.* Many of those parasites which selective oxicity seeks to destroy are primitive one-celled creatures vhich never carry out sexual reproduction (e.g. trypano-omes) or in which sexual reproduction does not seem to e of importance (e.g. bacteria). Or they may have sexual orms but do not reproduce by means of them in a given ost (e.g. malarial parasites in man). In such cases the ormal biochemistry of genetics is side-stepped. Again, he pharmacological applications of selective toxicity do ot involve genetic considerations.

2. *Carbohydrate metabolism.* Although the Meyerhof

sequence of glycolysis is very common, certain bacteria, yeast and fungi appear to be able to oxidize glucose directly; others oxidize glucose-6-phosphate directly. These methods, which are probably evolutionary relics, avoid the use of pyruvic acid and a good deal of evidence has been obtained lately that the *iso*citric acid cycle (Krebs' cycle) is imperfect in bacteria. Even when fermentation is known to proceed through pyruvic acid, strong specific differences are known. The transformation of this acid to alcohol is favoured by yeast, also by the higher plants and some bacteria, whereas other bacteria (and the majority of the higher phyla) transform it to lactic acid. In individual cases, yet other principal end-products of pyruvic acid fermentation are known.

Even where various organisms carry out identical tasks in the different stages of the glycolysis sequence, identical enzymes are not necessarily involved. For example, the hexokinase of malarial parasites has a lower sensitivity to inhibitors than that of yeast and red cells (Speck and Evans, 1945). The enzyme which makes oxalacetic acid from carbon dioxide and pyruvic acid in the liver is quite different in properties from the enzyme which performs this reaction in bacteria (Krampitz and Werkman, 1941; Evans, Vennesland and Slotin, 1943). The glucose-phosphorylases of muscle utilize adenylic acid as a co-enzyme, whereas those of yeast and potato do not. The alcohol-oxidase present in liver does not contain the active thiol-groups present in yeast alcohol-oxidase (Barron, 1943). The majority of aerobic pathways depend upon the intervention of the cytochrome system, but ten chemically distinct cytochromes are known, some of which are found only in bacteria. A few biological oxidations are known which by-pass the cytochrome system (e.g. the oxidation of glucose by *Penicillium notatum*).

Turning to phosphorylation, it is true that adenosine triphosphate is almost universally employed, but some primitive non-phosphorylative fermentation has been found

some bacteria, yeasts and other fungi (Barron, 1943). oreover, the phosphagens (secondary phosphorylating ents) differ between species in the very interesting way own in Table 1. It will be seen from this table that the e of creatine phosphate, as a phosphagen, is almost tirely confined to vertebrates or, at any rate, chordates, nereas arginine phosphate is used instead by invertebrates. otozoa have neither creatine nor arginine phosphates aldwin, 1948).

TABLE 1

DISTRIBUTION OF PHOSPHAGENS IN THE ANIMAL KINGDOM

| *Phyla* | *Arginine Phosphate* | *Creatine Phosphate* |
|---|---|---|
| lat worms . . | + | − |
| egmented worms | + | − |
| rthropods . . | + | − |
| Molluscs . . | + | − |
| rotochordates . | ± | ± |
| ertebrates . . | − | + |

Baldwin (1948).

3. *Fat metabolism.* Cells which are liable to be exposed ddenly to drying conditions usually have a high fat meta- olism, fat producing twice as much water as either carbo- ydrate or protein (Baldwin, 1948). Parasitic nematode orms afford a striking example of this biochemical eculiarity. Those who are interested in the chemotherapy f worm infections might find it useful to seek substances apable of blocking the metabolism of fats.

4. *Nitrogen metabolism.* The end-products of nitrogen etabolism display more variety than those of fat or rbohydrate metabolisms. These end-products range in mplexity from ammonia to the alkaloids. Table 2 ows the end-products of protein and purine metabolism mong vertebrates; it is no less varied among humbler

forms of life. Even within a single genus it can vary surprisingly, e.g. Dalmatian dogs convert their unwanted purines to uric acid just as human beings do, whereas other dogs convert purines to allantoin.

TABLE 2

END-PRODUCTS OF PURINE AND PROTEIN METABOLISMS IN VARIOUS PHYLA

| *Phyla* | *Protein Metabolism* | *Purine Metabolism* |
|---|---|---|
| Fish (teleosts) | ammonia | urea |
| Amphibia | urea | urea |
| Reptiles (snakes) | uric acid | uric acid |
| Birds | uric acid | uric acid |
| Mammals | urea | allantoin* |

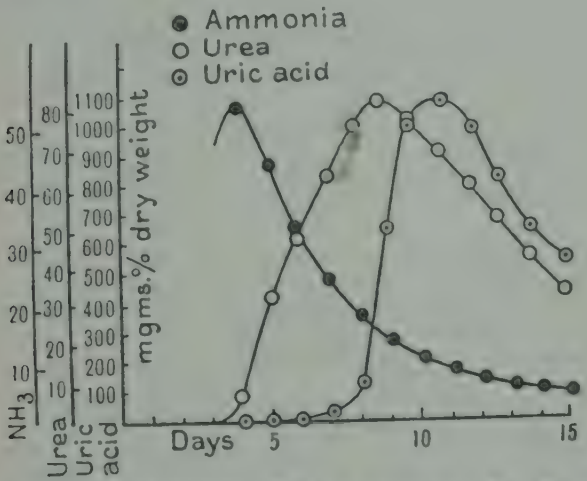
* Excepting man, higher apes and the Dalmatian dog, which excrete uric acid. Baldwin (1948).

What is still more surprising, the end-products of nitrogen metabolism can vary within a single species, depending on the stage of development. It can be seen in Fig. 2, for example, that a chicken, in the egg, passes in turn through the stages of excreting ammonia (as though it were a fish), urea (as though it were a frog) and uric acid (typical of birds), all within a few days. This sequence may shed light on the well-known necessity for using different selectively toxic agents to deal with parasites at different stages. For example, the sexual and the non-sexual forms of the malarial parasite, co-existing in the human blood-stream, are attackable with 'Atebrin' and 'Plasmoquine' respectively.

5. *Growth factors.* There may be a depressing similarity in the hormone requirements of various forms of life, but extreme individuality is often encountered where vitamins and minerals are concerned.

Rats and mice make their own vitamin C, but man and

guinea-pigs need to have it supplied in their food. Man manufactures all the vitamin D that he requires, provided that he lives in a climate where there are frequent periods of sunshine.




Nitrogen excretion of chick embryo (after Needham).

| Maximum of | Days |
|---|---|
| Ammonia | 4 |
| Urea | 9 |
| Uric acid | 11 |

Fig. 2.

(*Baldwin*, 1948.)

The fastidiousness of bacteria in their requirements for vitamins, amino-acids and trace-metals has been extensively studied. It is found that they differ enormously among one another in this respect.* Parasitic bacteria, in general, need a great variety of growth-factors supplied to them. Often, it is true, the same growth-factors are essential for their hosts, but in cases where this is not so, the

* For example, many bacteria can manage without an external source of pantothenic acid, but some strains of *Corynebacterium diphtheriae* need to be supplied with the β-alanine portion of this acid whereas other strains of this bacterium require the entire preformed acid. Likewise, many bacteria do not require an external source of nicotinic acid, but others require it to be supplied to them (as such) whereas fastidious bacteria require it supplied in the form of diphosphopyridine nucleotide.

way lies wide open for selective toxicity. One example that springs at once to mind is that of *p*-aminobenzoic acid, which is not essential for human beings but is very important for the majority of pathogenic bacteria. Many such bacteria can be severely injured without harming the patient by dosing him with sulphanilamide, which is a specific antagonist for *p*-aminobenzoic acid (see Chapters III and VII).

Bacteria stand apart from other forms of life in that their proteins contain an unique amino-acid: diaminopimelic acid (Work, 1950).

The requirements of trace-metals vary enormously from species to species. We need only think of the essential molybdenum requirements of nitrogen-fixing bacteria, the need for gallium* shown by the common mould *Aspergillus niger* (Steinberg, 1938), and the accumulation of vanadium by certain protochordates (the tunicates). The important part played by molybdenum in the nutrition of plants and fungi appears to have no counterpart in animal economy.

6. *Metabolism of foreign substances.* There are some surprising differences in the ways in which closely related genera handle foreign substances. For example, aromatic amines (e.g. aniline or sulphanilamide) are normally acetylated, and most species of fish, amphibia, reptiles, birds and mammals carry out this change. And yet amines are not acetylated by a few types of mammals (e.g. dogs), amphibia (e.g. certain species of frog) and reptiles (e.g. turtles).

Again, dogs and hens deal with quinoline by methylating it on the nitrogen atom, but rabbits insert a hydroxyl-group in the 6-position.

7. *Quantitative aspects.* So far we have been considering prominent qualitative differences in the biochemistry of species. But even where similar metabolic pathways are used by two species, quantitative differences become apparent.

* Gallium is not a rare metal: it is present in commercial aluminium and is scattered around the kitchen every time a saucepan is scraped.

or example, pathogenic trypanosomes utilize glucose two housand times faster than their hosts.* The carbo-ydrate metabolism of parasites is likely to be more ulnerable because less of it can be kept in reserve.

These quantitative differences do not always tell in favour f the larger creature. Man, for example, is fifteen times s sensitive to atropine as rabbits are. However, he can afely take a dose of strychnine which would kill more han his own weight of rabbits, and he is unaffected by a oncentration of hydrocyanic acid that is instantly fatal o dogs.

It follows from the various facts which we have just been eviewing that *comparative biochemistry* is, of all branches of science, the one that holds the master key for the logical iscovery of selectively toxic agents. It can reveal meta-olic differences between the economic species which man vishes to save and the uneconomic species which he wishes o destroy. Once these metabolic peculiarities are dis-covered, it should not be hard to devise selective agents vhich can use them to cause irreparable damage to the uneconomic species. Unfortunately, comparative bio-chemistry has so far attracted few workers. It is an unspectacular science and it is poorly endowed because it has often to concern itself with species of no *obvious* economic significance to man, although so much of selective toxicity is actually applied comparative bio-chemistry!

* Dr. E. M. Lourie has kindly supplied this figure, determined as follows: A million trypanosomes (*T. rhodesiense*) weigh 0·0078 mg., and consume 0·031 mg. of glucose in 5 hours. Hence, they consume 20 times their weight in 24 hours, but a man eats only 1/100 of his own weight in that period.

*CHAPTER TWO*

# (*a*) THE NATURE OF THE DRUG-RECEPTOR BOND. THE RELATIVE IMPORTANCE OF CHEMICAL AND PHYSICAL PROPERTIES IN SELECTIVE TOXICITY

# (*b*) STRUCTURAL NON-SPECIFICITY (FERGUSON'S PRINCIPLE)

# (*c*) HOW 'CHEMICALLY INERT' SUBSTITUENTS CAN INFLUENCE BIOLOGICAL ACTION

## (*a*) *The nature of the drug-receptor bond. The relative importance of chemical and physical properties in selective toxicity*

In this chapter a distinction will be made between two classes of selectively toxic agents: (i) those that are structurally non-specific and (ii) those that are structurally specific. The types of biological activity to be expected from a structurally non-specific substance is limited, but these substances provide some serviceable insecticides and most of the known hypnotics and general anaesthetics. On the other hand, structurally specific agents cover a wider range of biological action, generally act at greater dilutions and in many cases are more selective. The discussion of structurally specific agents will be continued throughout the remaining chapters.

An interesting introduction to this chapter is furnished by some easily performed experiments which illustrate chemical selectivity. Examples have been chosen from two branches of applied chemistry, viz. Dyeing and Flotation, which at first sight may seem to be remote from our main topic, selective toxicity. However, we should do well to look into branches of technology where a need has arisen for chemicals that are capable of making fine distinctions between rather similar substances. Such studies have more

common with our subject than meets the eye. We shall d that they help us to understand the problems that we e meeting in selective toxicity.

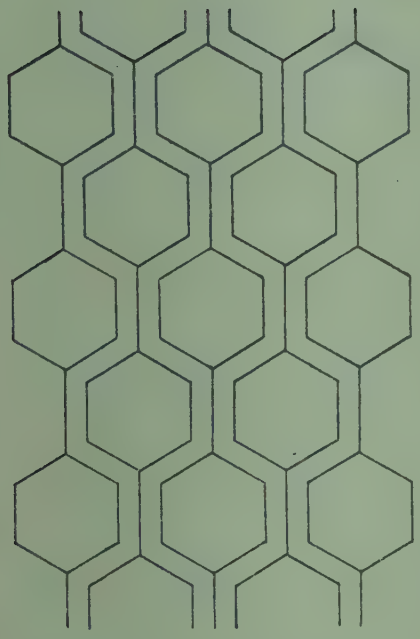


Fig. 1—Flat Structure of Cellulose.

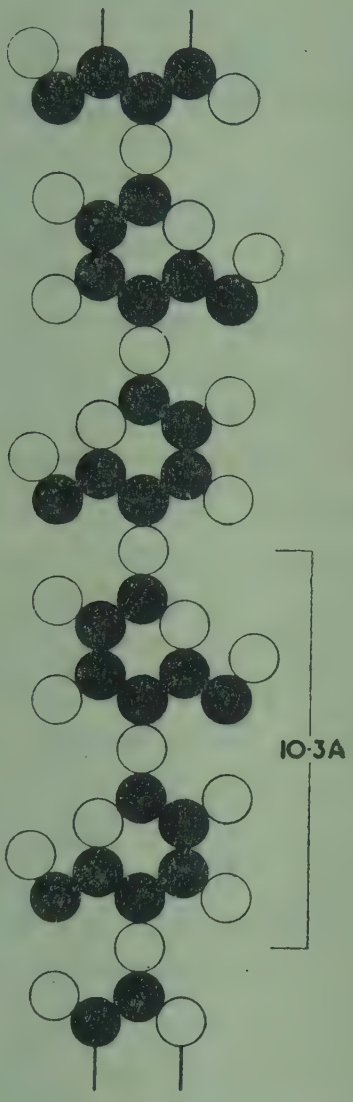



Fig. 2—Cellulose.

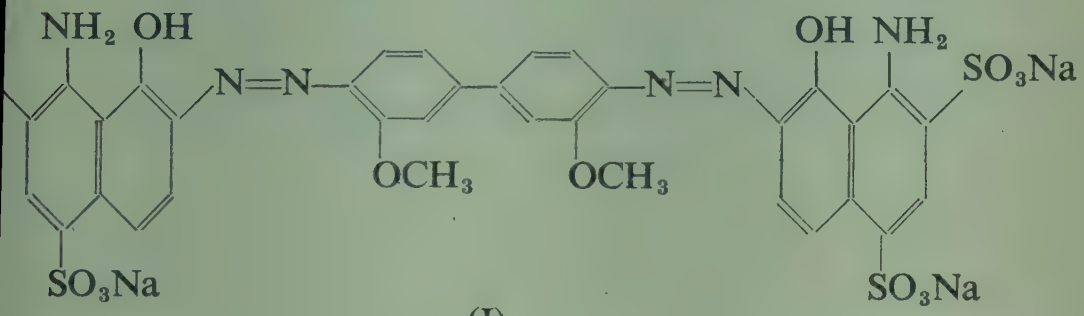
Our first experiment will demonstrate the dyeing of two hemically related fabrics, in contrasting colours, from the ne bath. Let us take a piece of cloth made of pure cellu-ose, and another piece of the same size and weight but nade of cellulose acetate. These two pieces of cloth may e stitched together. They should not be mordanted or retreated in any way but simply put into a dyebath and eated at 80° C. for ten minutes. The dyebath should

contain two simple azo dyes, similar in chemical constitution, in the proportions described in the Appendix to this chapter. One of the dyes, Chlorazol Sky Blue FFS (I), is very soluble in water and has an affinity for cellulose. The other dye, Dispersol Yellow 3G (II), is poorly soluble in water, but has a great affinity for cellulose acetate. Neither dye has any appreciable affinity for the other fabric. Hence when the fabrics are taken out of the dyebath, it is seen that the cellulose has become pure blue and the cellulose acetate pure yellow. In short, each fabric has combined very strongly with its appropriate dye and has rejected the opposite type. If we look at the structure of a cellulose fibre (Fig. 1) we see that it consists of long, rather flat molecules, packed together in sheets. Each molecule (Fig. 2) is liberally studded with hydrogen-bonding groups. Hence it is not surprising that the majority of water-soluble dyes which have an affinity for unmordanted cellulose are also long and flat and are liberally studded with hydrogen-bonding groups (Lapworth, 1940; Ruggli, 1934). Chlorazol Sky Blue is just such a substance. In cellulose acetate, however, five out of every six hydroxyl-groups have been blocked by acetylation and the molecule has taken on the general characteristics of an ester. Hence it is not surprising to find that the majority of dyes which have an affinity for cellulose acetate are insoluble in water but very soluble in esters. The union is no longer by means of hydrogen-bonding groups, and the possession of these is unimportant, or even disadvantageous (indeed, the introduction of a single sulphonic-group has been found to destroy all affinity for cellulose acetate).*

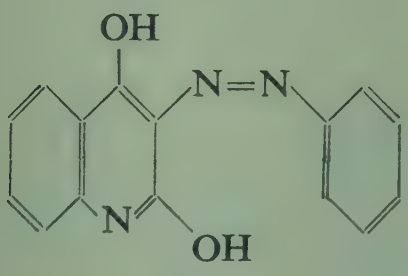
Our next experiment demonstrates the separation of a mixture of minerals by flotation. Red mercuric sulphide ore and green copper silicate ore (which have been crushed under water) should be placed in a cylinder. When we

* For further information on the connexion between constitution and selectivity in these dyes see Lapworth (1940) and Green (1937).

ake the cylinder and make a temporary foam, we observe at neither component of the mixture tends to rise with e air bubbles. If we now add as little as one part in 0,000 of potassium amyl xanthate (III) and shake again, e see that the mercury ore rises and leaves the copper ore the bottom. The xanthate has obviously functioned as



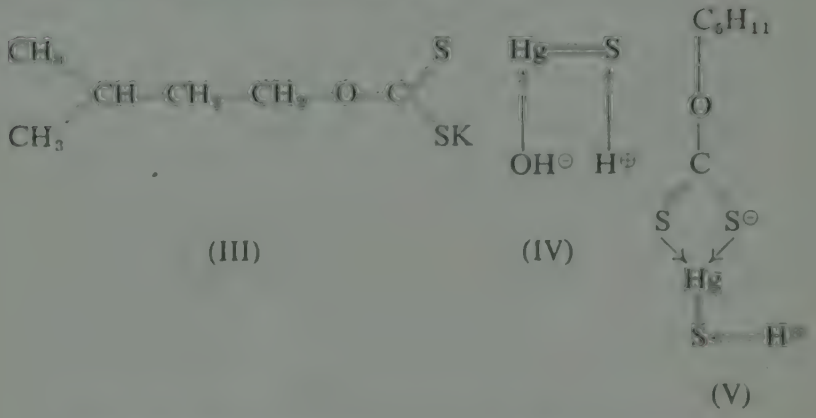

(I)




(II)

'collector', i.e. it has a specific affinity for the mercury om and successfully competes with the hydroxyl ions ith which it is combined. (Incidentally, we must bear in ind that new surfaces formed under water do not have e classical structures but are combined with hydrogen nd hydroxyl ions by ion-dipole bonds, as in IV). Now at the hydrophilic hydroxyl ions on the mercuric ore V) have been replaced by the hydrophobic xanthate ns (V), the particles of ore become attached to the air ubbles and rise with them. The xanthate ion has no finity for the copper ore whose particles remain uniformly etted and do not rise. The oleate and stearate ions, on e contrary, have great affinity for all metallic ores, nabling them to be separated from quartz. If we now

add a trace of soap to the cylinder and shake again, we shall see that both the mercury and the copper ores have floated up on the air bubbles.* This and similar processes are familiar to every mining engineer and are used commercially on an immense scale.



(III) (IV) (V)

These two experiments illustrate how chemicals can be found which possess such a high degree of selectivity that they can discriminate between chemically similar substances. The high degree of selectivity shown in chromatography by such substances as alumina and calcium carbonate provides a further example. No less striking is the rubber-latex impregnated bandage† which sticks to itself but not to skin. The more we study examples of selectivity in fields other than our own, the better we shall understand the true nature of the more difficult problems which selective toxicity poses.

It does not seem so very long ago that one heard a good deal of discussion as to whether the mode of action of selectively toxic agents was physical or chemical. It was even said sometimes that the physical properties of a drug were responsible for getting it to the site of action but that it would act by chemical means once it arrived there. By

* For further reading see Holman (1941), Wark (1938), Rogers and Sutherland (1947), Rogers, Sutherland, Wark and Wark (1946).

† e.g. 'Prestoband' (Vernon & Co. Ltd., Preston, Lancashire).

'chemical means', these debaters implied that either ionic or covalent bonds were formed.

How superficial this discussion now appears! From the standpoint of current knowledge we can no longer claim 'physical 'and 'chemical' as alternative sets of properties: rather, they are different aspects of the same set of properties. The physical properties which each chemical substance possesses are inevitable expressions of its chemical structure. To-day, nobody could limit the term 'chemical reaction' to the making and breaking of ionic and covalent bonds. On the contrary, it has become quite clear, during the last decade or two, that hydrogen-bonds, ion-dipole bonds and van der Waals' bonds play important parts in chemical reactions and that often quite comparable amounts of energy are involved. As Langmuir pointed out, even so simple an operation as the warming of water is actually a chemical depolymerization; the remarkably high specific heat of water is a measure of the strength of the hydrogen bonds which have to be broken. The success of the two very striking experiments which we have just observed depended on the formation of bonds which are neither ionic nor covalent.

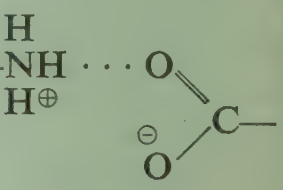
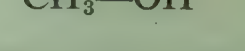
The principal types of bonds that we are likely to meet are illustrated on p. 26, and may be described as follows:

(i) *The van der Waals' bond.* This is the most universal of all bond types, because it is formed when *any two atoms* belonging to two different molecules happen to come near to one another. It is a very weak bond, increasing in strength with the atomic weight; hence it is probably of negligible strength where hydrogen is concerned, but it becomes significant for carbon, nitrogen and oxygen. As this bond can be formed only over a short distance, it requires a close fit between the two reacting molecules.*

* The attracting force for a van der Waals' bond is inversely proportional to the seventh power of the distance, i.e. if two atoms are removed twice as far from one another, the attraction falls to 1/128 of its former value. The attracting force for an ionic bond (see below) would only have fallen to ¼ of its former value.

TABLE 1

BONDS OF IMPORTANCE IN BIOLOGY

| *Bond types* | *Usual bond strengths* | *Example* |
|---|---|---|
| (i) Van der Waals' bond . . | *c.* 0·5 kcal. (?) |  |
| (ii) Hydrogen bond | 2 to 5 kcal. |  |
| (iii) Ionic bond . | 5 kcal. |  |
| (iv) Reinforced ionic bond . . | 10 kcal. |  |
| (v) Covalent bond | 40 to 100 kcal. |  |

The strength of attachment of a drug to its receptor by one of these bonds would be insignificant, but if the configuration of drug and receptor were so sterically similar that, say, a dozen such bonds could form, a really significant link would be forged (e.g. 5 kcal./mole). Alternatively a strong but non-durable bond, such as an ionic linkage can be made more permanent if reinforced with some van der Waals' bonds (see Chapter IV).

(ii) *The hydrogen bond.* This is a rather stronger bond than the above, and hence it does not require such an extremely close approach between drug and receptor. It is usually limited in biology to the linkage of two oxygen atoms (or one oxygen and one nitrogen) by a hydrogen atom. The attraction between an antigen and its antibody is said to consist entirely of short-range forces of the van der Waals' and hydrogen bond type (Pardee and Pauling, 1949).

(iii) *Ionic bonds* are formed between ions of opposite harge. They are also known as salt-linkages. As can e seen from Table 1, these bonds are quite strong. lowever, in a biological environment they lack perma- ence because of the large amount of simple inorganic alts present and the opportunities thus afforded for ion- xchange.

(iv) When an *ionic bond* is *reinforced* in some way it ecomes stronger and sometimes more permanent. For xample, the kations of all amines, except quaternary mines, simultaneously form hydrogen bonds and ionic onds with the anions of carboxylic acids. This doubles he strength of the bond. Again two molecules can be eld at one end by an ionic bond and elsewhere by van der Waals' bonds. This greatly increases the permanence of the bond.

*Ion-dipole bonds.* Many molecules which are not ion- zed carry fractional positive and negative charges on some of their constituent atoms. These charges can attract ions of the opposite sign, thus forming weak bonds which are probably comparable with van der Waals' bonds in strength. Few examples of this type of bond have yet been detected in biology (IV and V are examples from mineral chemistry).

(v) *Covalent bonds*, in which electrons are actually shared between atoms, are the typical bonds of the organic chemist who spends so much of his time making and breaking them. They are usually much stronger than the other types of bonds just described and for this reason they are believed to be seldom responsible for the union of a selective toxic agent to a receptor. The reason for this belief is that toxic action is reversible in so many cases, when experimental conditions are changed. Now, the greatest strength of a bond which can be readily broken at room-temperature or body-heat is about 10 kcal. The arsenic-sulphur bond is an example of a covalent bond that is sufficiently weak, but the majority of covalent bonds are

too strong. We shall discuss the covalent bond further in Chapter III.*

Most of the remaining chapters are concerned with selectively toxic agents whose action depends upon considerable specificity of structure. Such substances need to have particular molecular outlines or certain reactive groups in order that they may combine with specific cell constituents. However, there is one class of agents which is not structure-specific in this way, and we shall consider it first of all.

(*b*) *Structural non-specificity* (*Ferguson's Principle*)

It has long been known that the action of hypnotics, general anaesthetics and some of the milder insect-poisons is not to be related to the presence or absence of some particular chemical group, but depends rather on certain physical properties which make for accumulation. Such substances may be hydrocarbons, chlorinated hydrocarbons (aliphatic and aromatic), alcohols, ethers, ketones, non-ionized bases (such as aniline and pyridine) or non-ionized acids (particularly phenols and barbiturates) or aliphatic nitro-compounds but probably not aldehydes and esters and certainly not (*inter alia*) strong bases and acids. Structurally non-specific agents seem to act by accumulating in some vitally important part of a cell and thus disorganizing the chain of respiratory processes. In short, they are acting simply as foreign bodies. Obviously they owe their property of being accumulated by cells to some favourable partition-coefficient which they possess, a physical property which can only be conferred upon them by their chemical constitution. Hence it is undesirable to speak of these substances acting 'by a physical mechanism' in contrast to the action of structurally specific substances. However, it is obvious that the required partition coefficients depend only on the balance between two, or at the

* For further information on the various types of bonds see Pauling (1946, 1947).

most three, common variables in molecular structure so that the correct values will be reached by certain members of homologous series of many different chemical types. The biological action of these structurally non-specific substances is usually depressant (narcotic).

In their classical studies of structurally non-specific narcotics, Overton and Meyer believed that the action depended upon a suitable partition-coefficient between water and lipoid material (cf. Höber, 1945). This hypothesis does not lend itself to experiment because there is no guide to the correct lipoids to choose for laboratory studies. Certainly the choice of olive oil for this purpose by the early workers seems wrong from what we now know of the nature of the water-insoluble constituents of cells (phospholipides and fatty alcohols). Furthermore, the choice of water as the non-oily phase would exclude the discussion of insecticides administered in air.

Fortunately, a way out of this difficulty exists. Ferguson (1939) suggested that the partition of the toxic agent between the medium in which it was applied and the biophase* in which it is acting would always be proportional to the saturation of that medium, whether air or water. This hypothesis was derived from thermodynamic considerations and involves approximations whose validity cannot be assessed. However, it has a corollary which is capable of experimental verification, namely, *substances which are present at the same proportional saturation in a given medium have the same degree of biological action.* This, Ferguson's Principle, has been experimentally examined and seems to be true of all structurally non-specific substances (Ferguson, 1939; Ferguson and Pirie, 1948; Burtt, 1945; Gavaudan, Dodé and Poussel, 1944).

As an example of Ferguson's Principle, we may consider the effect of various hydrocarbons, halogenated paraffins, nitro-hydrocarbons and weak bases on the wireworm (larva of *Agriotes*) (Table 2). These substances were applied as

* i.e. the relevant cell-constituent, apparently lipoidal in nature.

TABLE 2

AGREEMENT BETWEEN THE RELATIVE HUMIDITIES OF THE TOXIC CONCENTRATIONS OF VARIOUS SUBSTANCES TESTED ON WIREWORMS

| *Substance* | *Toxic concentration, millionths g. mol. per l. lethal in* 1,000 *min. at* 15° C. | $P_s$ (*Vapour pressure at* 15° C. *mm.*) | $P_t/P_s$ (*Relative humidity of toxic concentration.*) |
|---|---|---|---|
| Monomethylaniline | 3·7 | 0·22 | 0·3 |
| Dimethylaniline | 6·6 | 0·28 | 0·4 |
| Pyridine | 76 | 10·4 | 0·1 |
| Bromoform | 94 | 3·2 | 0·5 |
| Bromobenzene | 96 | 2·4 | 0·7 |
| Tetrachloroethane | 141 | 4·2 | 0·6 |
| Chlorobenzene | 200 | 6·8 | 0·5 |
| *p*-Xylene | 230 | 7·0 | 0·6 |
| Toluene | 420 | 17·0 | 0·4 |
| Nitromethane | 710 | 23 | 0·6 |
| Benzene | 775 | 58 | 0·2 |
| Heptane | 800 | 27 | 0·5 |
| Chloroform | 1,040 | 128 | 0·2 |
| Carbon tetrachloride | 1,600 | 73 | 0·4 |
| Trichloroethylene | 1,200 | 52 | 0·4 |
| Hexane | 3,000 | 96 | 0·6 |
| Dichloroethylene | 3,100 | 230 | 0·2 |
| Pentane | 16,600 | 320 | 0·9 |

vapours, diluted with air. It is seen from Table 2 that when equimolar concentrations are compared (as in column 2), a 4000-fold variation in activity is seen. However, a different picture presents itself when the proportional saturations are compared, e.g. by calculating the least fraction of the saturated vapour pressure required to kill the worm. Here (within the much narrower limits of a ninefold variation) a reasonably constant figure (0·5) is obtained (Ferguson, 1939). That is to say, the air must be half-saturated. On the other hand, hydrocyanic acid,

bon bisulphide, ammonia and strong organic bases
e non-concordant figures (e.g. ammonia, 0·00008).
Were the figure 0·5 a constant for the action of structur-
non-specific substances on every kind of cell, it would
idle to expect them to show any selectivity in their
icity. However, this figure varies, for different cells,
ween 0·01 and 1. It is approximately 0·03 for the
duction of narcosis in mice and tadpoles and for the
ibition of the development of sea-urchin eggs. The
ue 0·5 has been found for the minimal concentrations of
iline, phenols, aliphatic alcohols and ketones required
kill *B. typhosus* in aqueous solution, i.e. approximately
f-saturated solutions are required (Ferguson, 1939).
The great value of this principle to those who are working
th new toxic agents is that one can so quickly find out if
eir action is structurally non-specific or not. First one
ould determine the Ferguson Value for the particular
ological effect that one is studying, using a dozen or so
amples of the above-mentioned chemical types. If the
w drug, when tested, gave a similar figure, the likelihood
its being structurally non-specific is high. Hence one
ould not expect it to show particularly novel biological
operties and would not map out a programme in which
e molecule was to be altered in minute particulars, e.g.
inserting a methyl group in various positions.
Another utilitarian conclusion can be derived from the
rguson Principle, viz. it can be extraordinarily econo-
cal, when investigating a new biologically active chemi-
, to alter the molecule so that it becomes less soluble in
ter. However, there is a limit to the extent to which one
n profitably go in this direction, as will be pointed out
er in this chapter.

### *How 'chemically inert' substituents can influence biological action*

Until comparatively recently, difficulty has been found
understanding how two molecules which differ from one

another only by a methyl group sometimes have biological actions of an entirely different order. We shall shortly be considering several examples of this kind. It is true that in those cases where the methylated compound is the less active, the reason may be that the methyl group is oxidized to a carboxylic acid group thereby completely changing the distribution of the substance (cf. Schultzen and Naunyn, 1867). However, it is just as common for the methylated compound to be the more active. Hence we should consider at some length how a methyl (or methylene) group could alter the biological action of a molecule without involving covalent bonds. Once we understand the action of these groups, we can modify our findings and apply them to many other kinds of substituents, e.g. the halogens, which seem, in many instances, to act without involving covalent bonds.

There come to mind at least four main channels through which methyl groups can exert a profound influence on biological activity:

Firstly, by effecting a change in solubility, which can be either raised or lowered;

Next, by steric effects which can either increase or decrease the biological action;

Thirdly, by effects on the oxidation-reduction potential which is lowered by the electrons poured into the molecule through the inductive effect of the methyl group;

Lastly, by the similar inductive effects on the ionization constants, resulting in a weakening of acids and a strengthening of bases.

I should now like to discuss various biologically active substances in which one or other of these effects seems to be at work.

Obviously the simplest case of an inert substituent exerting a biological action is to be found in homologous series. In ascending an homologous series, one may find that the first member shows some irregularity, but from there onwards each member is found to be more biologically active than the last. Nevertheless, higher in the series an

different places in it for different tissues or organisms, oxicity is completely abolished by the addition of just one ore $-CH_2$ group. Now, it is not surprising that toxicity hould increase as a series is ascended because the addition f each methylene group makes possible the creation of nother van der Waals' bond, thus adding to the adsorptive orces which bind each substance to the organism's recep-ors. At the same time, there is no increase in the desorptive orces, because the kinetic energy of every molecule is dentical, regardless of its size.

However, the sharp cut-off in biological activity, occur-ing as it does at different members of the series in different iological tests, presents a harder problem to solve. Ferguson (1939) first explained this puzzling phenomenon y showing that, in an homologous series, the equitoxic oncentrations for various members usually fall on a straight ine, if plotted logarithmically: solubilities also fall on a traight line, but these two lines are not quite parallel and ence they intersect. It is at those points where the solu-ility and equitoxic-concentration lines intersect that the harp cut-off occurs.

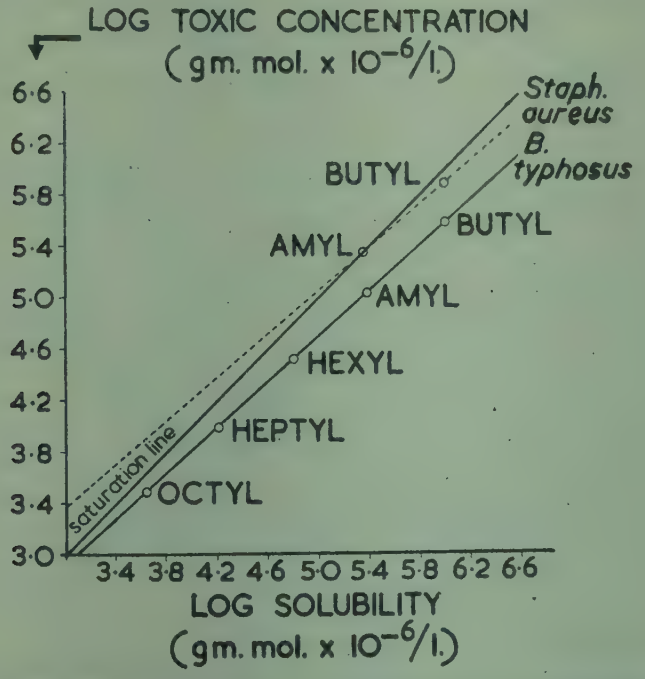



Fig. 3—Bactericidal concentration *v.* solubility for normal primary alcohols.

This is clearly shown in Fig. 3, where the toxic concentrations of primary alcohols have been plotted against their aqueous solubilities. It can be seen that the Gram-negative organism *B. typhosus* is very sensitive to these alcohols so that, even as high up in the series as octanol, the required lethal concentration is not in excess of the solubility. On the other hand, the Gram-positive organism *Staphylococcus aureus* is less sensitive to alcohols, so that a higher concentration is required for killing. Consequently a sharp cut-off occurs at amyl alcohol because the predictable lethal concentration of hexanol is in excess of the solubility (cf. also Badger, 1946). In Fig. 3, the 'saturation line' is a diagonal, i.e. the plot of log. solubility *versus* log. solubility. It is the line on which would fall the points for an imaginary series of substances that were optimally active in saturated solutions.

It might be thought that the insertion of a methyl- or methylene-group, being water-repelling, would always lower solubility in water, but this is not necessarily so. Even isomerides can differ greatly in solubility, e.g. the isomeric pentanols (Ginnings and Baum, 1937) in Table 3. It can be seen that the solubility increases as the side-chain is broken up into smaller lengths. This is only to be expected, because the most characteristic property of water is the strong hydrogen-bonding that exists between its molecules. For a substance to dissolve, the water molecules must first be forced apart, which means that these bonds have to be broken. This is readily accomplished by the lower alcohols, because their hydroxyl-groups, which are also capable of hydrogen-bonding, form such a high proportion of the molecules. But as the series is ascended, the paraffinic side-chain becomes a more dominant feature: it cannot be accommodated in any interstices, it cannot force the water-molecules apart, and hence it tends to be squeezed out of the water, dragging the whole molecule with it. This effect is considerably lessened by shifting the hydroxyl-group to the centre of the molecule (as in tertiary amyl

cohol) and it is interesting to see that this particular ntanol is even more soluble than *n*-butanol. Again the amino-butyric acids are more soluble than α-amino-opionic acid (alanine) (Cohn *et al.*, 1934).

The sulphonamido-pyrimidine drugs constitute a series here the addition of methyl-groups strikingly increases

TABLE 3

SOLUBILITIES OF ALCOHOLS
(g. per 100 g. WATER AT 20°)

| | |
|---|---|
| ENTANOLS | |
| $CH_3—CH_2—CH_2—CH_2—CH_2—OH$ | 2·4 |
| $CH_3—CH_2—CH_2—\underset{\displaystyle OH}{\underset{\vert}{CH}}—CH_3$ | 4·9 |
| $CH_3—\overset{\displaystyle CH_3}{\overset{\vert}{\underset{\displaystyle OH}{\underset{\vert}{C}}}}—CH_2—CH_3$ | 12·2 |
| -BUTANOL (for comparison) | 8·2 |

olubility (see Table 4). At first sight this seems surprising ecause these substances are acids and the addition of each ethyl-group decreases the ionization of the acidic $SO_2NH—$) group. This decrease in ionization is just what ould be predicted from the inductive effect of the methyl roups which radiate electrons to the —NH bond, thus creasing the attraction between hydrogen and nitrogen. Vhat would, perhaps, not be predicted is that sulphadia-ne should be less soluble than its methyl-derivatives, if nly because sulphadiazine is the most ionized member of e series and it is normal for an ion to be more soluble an the corresponding neutral molecule. No explanation as been put forward for this abnormal effect of methyl

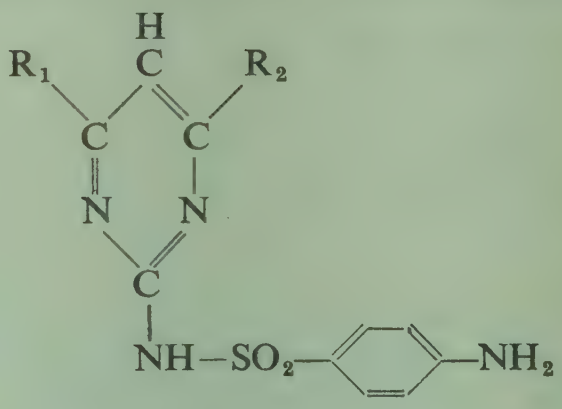
groups, but it is to be found in other molecules having a similar degree of complexity and rigidity. It may be due to the protruding methyl-groups preventing the ready adsorption of dissolved solute on to the crystal-lattice of the solid phase, thus displacing the final equilibrium in the direction of increased solubility.

The solubility measurements in Table 4 were made at pH 5·2, because this has practical significance in urinary disinfection and also in assessing the ease of clearance of the drugs by the kidneys* (Gilligan and Plummer, 1943). The same solubility sequences were shown at pH 6 and 7.

The loss of biological activity in the upper part of an

TABLE 4

INCREASED SOLUBILITY IN WATER CAUSED BY INSERTION OF METHYL GROUPS



| $R_1$ | $R_2$ | *Drug* | *pK (acidic)* | % *Ionized at pH 5·2* | *Solubility at pH 5·2 g. mol./l. (37° C.)* |
|---|---|---|---|---|---|
| H | H | Sulphadiazine | 6·5 | 3·9 | 0·0005 |
| $CH_3$ | H | Sulphamerazine | 7·1 | 1·4 | 0·0013 |
| $CH_3$ | $CH_3$ | Sulphamezathine | 7·4 | 0·7 | 0·0024 |

* All three drugs are less soluble than their acetyl derivatives, otherwise the solubility of the latter would be of special relevance.

mologous series can also have a slightly different cause
m that shown in Fig. 2. The primary aliphatic amines,
example, have solubility and toxicity curves which are
equately spaced. Nevertheless, for most bacteria, a
aximum in toxicity is reached somewhere about the $C_{12}$
ember (dodecylamine) and a rapid falling off occurs
nen one or two more carbon atoms are added to the long
raffinic side-chain (cf. Fuller, 1942). It should be noted
at this falling-off occurs in that part of the series where
icelle-formation is beginning to increase rapidly with
crease in chain-length. Thus the critical micelle concen-
ation falls from 0·01 to 0·003 in passing from the $C_{12}$ to
e $C_{14}$ amine (Klevens, 1948), and thus each higher
omologue contributes fewer molecules of monomer, even
moderately dilute solutions. If we assume (and this is
ery likely) that the bacterial receptors and the micelles are
competition with one another for the unassociated
olecules, it is easy to understand how this decreasing
oncentration of monomer can cause the known falling-off
biological activity.

Let us now turn to some useful examples of structural
ecificity to be gleaned from the vitamins. We shall have
casion later to refer to the theory that many drugs act
y blocking normal enzyme action in cells. At this stage
is relevant simply to point out that those vitamins which
rm part of co-enzymes have a high degree of structural
ecificity (cf. McIlwain, 1942). In fact, the presence or
sence of a methyl-group in these substances can make
mar their biological activity.

In the case of nicotinamide (VI), for instance, the
tivity completely disappears if it is methylated in the
position. This may be a purely steric effect, but the
ssibility that it is caused by a lowering of the oxidation-
duction potential should also be considered. Anyone
ho has attempted the polarography of pyridines will know
w extremely difficult they are to reduce: one may finally
ply a potential sufficient to electrolyse the water without

effecting the reduction of the majority of pyridines (including nicotinamide). However, nicotinamide occurs naturally as a nucleotide with ribose, adenine and pyrophosphoric acid. It is attached to the ribose in such a way that the ring-nitrogen is quaternary, an arrangement that just suffices to make the nucleotide reducible enough to act as the vitally important hydrogen carrier that it is. When this co-enzyme accepts hydrogen, the double bond in the 2-position is reduced. Now, it is evident that a methyl-group in the 2-position would, by its inductive effect, be transmitting electrons into this double-bond and thus hindering a reduction which is already difficult enough.*

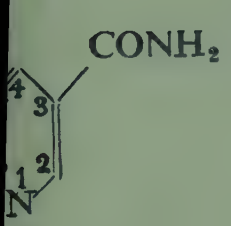
Heteroauxin (VII), one of the natural growth-regulators of plants, is another example of a substance whose powerful biological action is abolished by the insertion of a methyl-group in the 2-position.

On the other hand, in the K group of vitamins (e.g. menaphthone, VIII), the presence of a methyl-group (in the 2-position) is absolutely essential for biological activity.

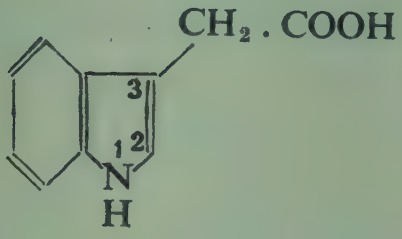
Thiamine (IX) provides examples of both types of effects. If the methyl-group is removed from the 2-position in the pyrimidine ring, the vitamin-activity (as measured on pigeons) drops to 1/22 of its former value. If a methyl-group is removed from the 4′-position of the thiazole ring, activity drops to 1/82, and if it is inserted into the 2′-position of that ring, the activity completely disappears. Thiamine, in the form of diphosphothiamine, forms an important portion of the enzyme system catalysing the decarboxylation of pyruvic acid, but it is generally considered not to undergo oxidation-reduction changes in discharging these functions. Here, at least, the effect of the methyl-group seems to be best explained on steric lines, namely that (depending on its position) it improves or worsens the fit between the vitamin and the rest of the enzyme-system.

* For the quantitative effect on oxidation-reduction potentials of various substituents see Fieser and Fieser (1935).

The case of folic acid (X) suggests that a redundant thyl-group may act, not so much by making it difficult a co-enzyme to combine with its apo-enzyme (i.e. its ecific protein), but rather by interfering sterically with




(VI)

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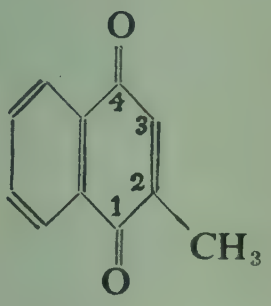


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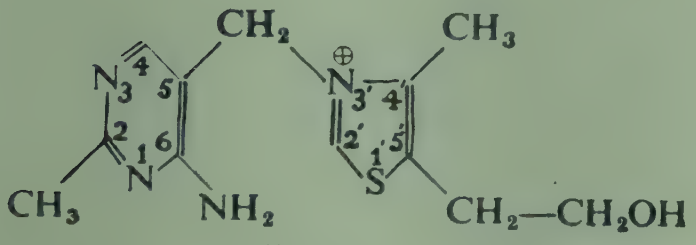


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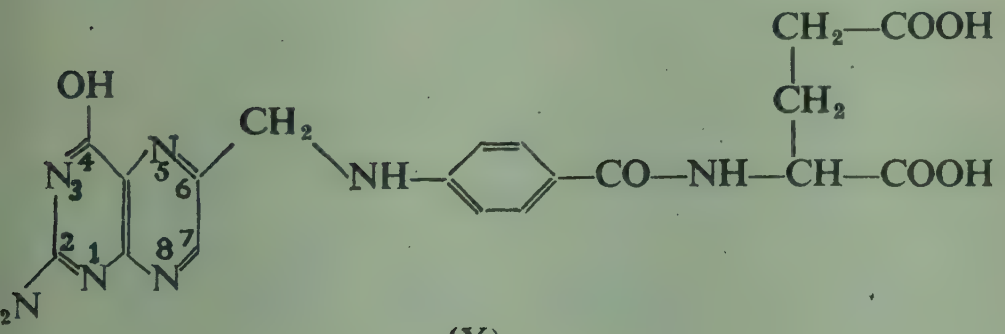


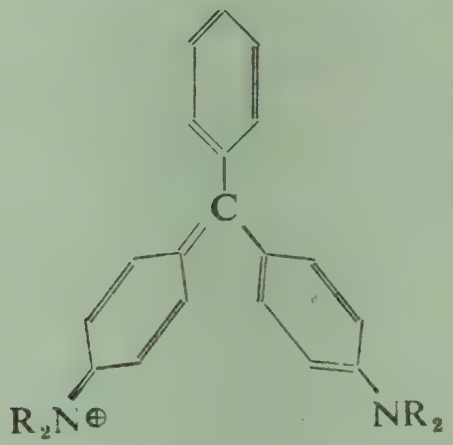

(X)

e working of that combination. The insertion of a ethyl-group into the 7-position of folic acid produces substance which can antagonize the normal biological tion of this growth-factor. This is one of the few known ses where the substitution of a methyl-group for a drogen atom has led to the production of a metabolite tagonist.

The antibacterial effect of 8-hydroxyquinoline, which will be discussed in Chapter V, is considerably depressed by the presence of a methyl-group in the 2-position and all the indications are that this group presents a degree of steric hindrance to proper adsorption. The effect of methyl-groups in conferring cancer-producing properties on polycyclic hydrocarbons will be discussed in Chapter VIII.

TABLE 5

CONNEXION BETWEEN IONIZATION AND ANTIBACTERIAL ACTIVITY IN A SET OF TRIPHENYLMETHANE COMPOUNDS




| *Substance* | *R's (all four)* | *pKa (equil.)* | *Per cent Ionized pH 7·3* | *Min. Bacteriostatic conc'n. for* Staph. aureus, *24 hours at 37° C. and pH 7·3* |
|---|---|---|---|---|
| Doebner's Violet | H | 5·38 | 2 | 1 in 20,000 |
| Malachite Green | $CH_3$ | 6·90 | 28 | 1 in 80,000 |
| Brilliant Green | $C_2H_5$ | 7·90 | 80 | 1 in 1,280,000 |

We have already had occasion to note how the inductive effect of a methyl-group decreases the strength of acids.

nversely, it increases the strength of bases when substi-
ted on a carbon atom, and in some cases it even increases
sic strength when substituted on a nitrogen. These
erations in strength are small (usually less than one pK
it) but become important when the pK of the drug is
thin 1 unit of the pH at which the biological test is made.
his is explained in Chapter IV. It will suffice to say here
at a shift of 1 pK unit, under these circumstances, can
ing about a fivefold increase in ionization. When, as
ten happens, one form of the drug (say, the ion) is
ologically active and the other form (say, the neutral
olecule) is not, this change in ionization can be a limiting
ctor which decides whether the substance will be biologi-
lly active or not.

An example of increase in basic strength induced by
-alkylation is taken from the triphenylmethane series
Goldacre and Phillips, 1949). It can be seen from Table 5
at antibacterial activity is strongly correlated with ioniza-
on for the substances examined and that the antibacterial
ctivity depends on the presence of 'chemically inert
roups'.

# APPENDIX

## (To CHAPTER II)

### *Experiments illustrating Selective Adsorption*

1. *Selective Dyeing*

Sew a piece of viscose material (about 180 square inches in area and 20 g. in weight) to a similar piece of cellulose acetate material.

Make a solution of Chlorazol Sky Blue FFS by dissolving 0·5 g. in 100 ml. of boiling water.

Prepare a suspension of Dispersol Yellow $3G_{300}$ by making 0·5 g. into a smooth paste with a little water, diluting to 50 ml., adding a non-ionic dispersing agent (e.g. 8 ml. of Dispersol VL) and making up to 100 ml.

Prepare the dyebath by mixing 25 ml. of each of the above solutions with a litre of water at 85° C. containing 8 g. of sodium chloride and 0·5 g. of an anionic wetting agent (e.g. Teepol).

Immerse the cloth in the dyebath and gently stir, well submerged for 10 minutes at 80°. (The experiment may be ruined if part of the cellulose acetate reaches 90°, because of hydrolysis to cellulose.)

Transfer the cloth, by means of glass rods, to a litre of cold water, squeeze well and spread out to dry. It will be found that the viscose has been dyed pure blue and the cellulose acetate pure yellow.

2. *Mineral Flotation*

Clean all apparatus used in this experiment with chromic acid, because a trace of grease will cause both minerals to float.

Crush red mercuric sulphide ore (cinnebar) and green cupric silicate, separately, in a mortar *under water* and pass each suspension through a sieve (70 mesh). The sifted suspensions should be freed from very fine particles by decantation. The solids must never be allowed to become dry from the moment of crushing until the end of the experiment.

Add about 1 g. of each mineral to a stoppered (100 ml.) cylinder containing 100 ml. of water and 0·1 ml. of amyl alcohol. To one of two such cylinders add 1 mg. (not more) of potassium amyl xanthate.

Shake both cylinders violently, by hand in a horizontal plane, for a few minutes and then stand them upright. It will be seen that, in the cylinder containing the xanthate, the red mineral has floated, leaving the green mineral on the bottom. In the other cylinder, both minerals remain on the bottom.

*CHAPTER THREE*

# METABOLITE ANALOGUES
# THE COVALENT BOND IN RELATION TO SELECTIVE TOXICITY: THE ARSENICALS AND MERCURIALS

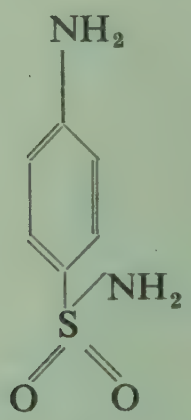
the previous chapter a number of cases were reviewed which the smallest change in the chemical constitution a selectively toxic agent made a very radical change in biological activity. In one case (folic acid) the biological tivity was completely reversed. The present chapter tiates a discussion of those toxic agents which can lectively antagonize particular metabolites. It begins th an account of those antagonists which are chemically nilar to the metabolites whose biological effects they ppress.

## *Metabolite analogues*

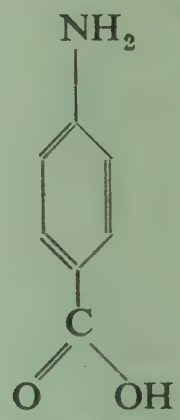
By *metabolite* we understand any substance which is volved in the biochemical reactions by which cells assimi- e food and use the resultant energy for growth and vision. The most important metabolites, for our purpose, e those which are present in the smallest amounts: it ould be easier to antagonize these than such metabolites are present more abundantly (e.g. the glucose-phos- ates). Hence we shall be concerned mainly with catalytic bstances such as hormones, vitamins and trace-metals. *metabolite analogue* we understand a substance which fers from a metabolite only by a small degree of chemical ange. Usually, if one makes a slight alteration to the emical structure of a metabolite, the normal biological ion is diminished or lost, as we saw in the last chapter, t antagonistic action does not invariably appear. Later, shall review the changes in structure likely to turn a

metabolite into an antagonistic substance.* It is with *antagonistic* metabolite analogues only that we are now concerned.

Perhaps the most familiar example of a metabolite analogue is sulphanilamide (I), which antagonizes the action of the chemically similar substance, *p*-aminobenzoic acid (II), which is a metabolite of great importance to micro-organisms. As is usual with metabolite analogues, this antagonism is competitive,† that is to say, if $x$ molecules of metabolite are antagonized by $y$ molecules of analogue, then $2x$ molecules of metabolite will require $2y$ molecules of analogue to give the same quantitative result, and so on. Such competitive reactions are freely reversible so that, in our hypothetical example, the antagonism of $x$ molecules of metabolite by $y$ molecules of analogue could be abolished by the addition of another $x$ molecules of metabolite, and so on.




(I)




(II)

Many metabolite analogues are known; Woolley (1947) lists forty metabolites for which antagonistic analogues have been made. For example, pyrithiamine, which was first made in 1941, has proved to be a competitive

* Occasionally, a big change in an unimportant part of a metabolite leaves the activity unimpaired, but this is rare. The loss of the hydrocarbon side-chain of natural Vitamin K, to give menaphthone, is an example.

† This is usual, but not invariable: 2:3-Dichloronaphthoquinone antagonizes the action of menaphthone (2-methylnaphthoquinone) in fungi. Over a narrow range of concentrations, this is competitive; but no amount of menaphthone will reverse a large dose of the dichloro- compound.

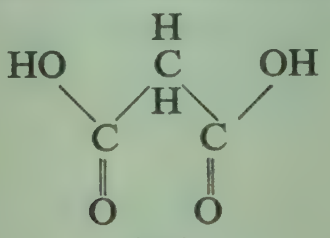
tagonist of the vitamin thiamine. It produces the symp-
ms of thiamine deficiency in mice much faster than
n be accomplished by feeding mice on a thiamine-free
et (Woolley and White, 1943). The effect of 100 lethal
oses of pyrithiamine can be annulled by increasing
e thiamine content of the diet 1,000-fold. Apart
om its action on mammals, pyrithiamine inhibits the
owth of yeasts, moulds and bacteria. Metabolite ana-
gues have since been found which are capable of antago-
izing, respectively, ascorbic acid, biotin, folic acid,
icotinic acid, pantothenic acid, pyridoxine, riboflavine,
ocopherol and vitamin K as well as most of the amino-
cids, the purine bases and one or two hormones. Some
f the toxic effects of metallic ions can be attributed to
heir antagonism of inorganic metabolites (see Chapter V).

There are some excellent reviews dealing with metabolite
nalogues (e.g. Work and Work, 1948, Chapter V; Woolley,
946 and 1947; Roblin, 1946).

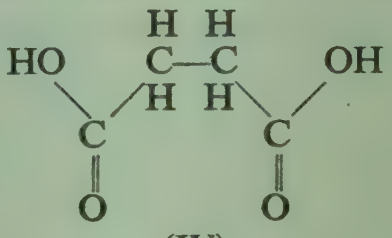
As far as chemotherapy is concerned, there was no
ecognition of the important part which metabolite ana-
ogues could play in the cure of infectious diseases until
ildes (1940) pointed out that the relationship of sulphanil-
mide to *p*-aminobenzoic acid was of this kind. Fildes
ave an impetus to further research along these lines by
uggesting that other metabolite analogues, useful in
hemotherapy, would eventually be found.

In other branches of biological science, however, the
henomenon of competitive inhibition by metabolite ana-
ogues had been recognized for many years. For example,
has long been known that enzymes are inhibited by
ubstances that are structurally related to the normal
ubstrates of these enzymes. Thus, amylase, an enzyme
hich turns starch to maltose, is inhibited by glucose
Wohl and Glimm, 1910). Again, malonic acid (III)
ompetitively inhibits the enzyme succinic dehydrogenase
VI), by displacing the normal substrate, succinic acid
V) from the enzyme which normally converts succinic

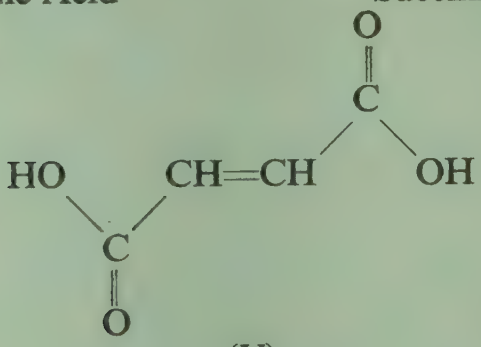
acid into fumaric acid (V).* *d*-Histidine similarly inhibits the enzyme histidase which normally acts on the *l*-isomeride (Edlbacher, Baur and Becker, 1940). Similar phenomena have been recognized in physiology ever since it was found that the toxic action of carbon monoxide (C—O) is due to its displacing a similarly shaped molecule, oxygen (O—O), from combination with haemoglobin (Douglas, Haldane and Haldane, 1912). In pharmacology, the competition of acetylcholine (metabolite) and physostigmine (analogue) for an enzyme (cholinesterase) has been known for many years (Loewi and Navratil, 1926; Stedman, 1926), and Stedman and Aeschlimann lost no time in simplifying the structure of physostigmine so that only the bare essentials were left. (See Table 1, p. 48.)



(III)
Malonic Acid

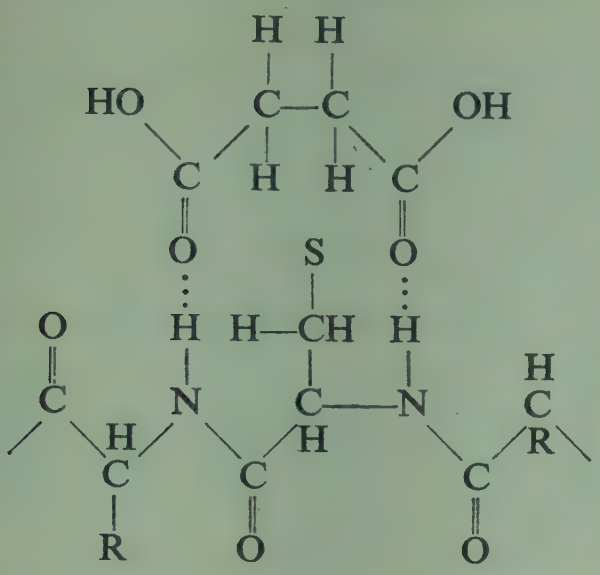


(IV)
Succinic Acid



(V)
Fumaric Acid

* The 'active-patch' of succinic dehydrogenase (VI) has been drawn from a suggestion made by Potter and Dubois (1943). These authors give reasons for believing that succinic acid is anchored by its two carboxyl-groups to two amido-groups of the peptide backbone of this enzyme, the linkage being by means of hydrogen bonds. As fast as the cytochrome system, which is always closely associated with this enyzme, can remove the hydrogen atom from the thiol group, this group removes an atom of hydrogen from the succinic acid. Two atoms of hydrogen are successively removed in this way, giving fumaric acid which, having a *trans*-configuration, is bent away from the enzyme. Thus it becomes detached and another molecule of succinic acid takes its place, and so on. Of all enzymes, this is perhaps the one whose active patch has been most clearly visualized.



(VI)

Suggested configuration of active patch of succinic dehydrogenase, with adsorbed succinic acid.

t is not surprising that there is now a very widespread ief that almost all drugs and other selectively toxic stances will be found to exert their ultimate biological ct by interfering with the working of enzymes. How-r, as we have just seen, there is at least one toxic stance that does not do so, viz. carbon mon-de.*

t is appropriate to point out here that there are many s of interfering with an enzyme system other than by competitive antagonism of metabolite analogues. For ance, substances which do not resemble metabolites at can be used to combine with, and hence inactivate, an -enzyme, a co-enzyme or a substrate, provided that e chemical affinity exists. What distinguishes the ial case of the metabolite analogue is that it brings ut 'antagonism by displacement'. To do this, its ecular architecture must be so similar to that of the strate that the enzyme is deceived into taking it up in

Haemoglobin is not an enzyme because it does not activate its substrate. nly a carrier which discharges oxygen in the same form as that in which aken up, viz. in the molecular state.

## TABLE 1

COMPETITIVE METABOLITE INHIBITION

| *Investigators* | *Protein* | *Inhibitor* | *Metabolite* | *Inhibitory Index** |
|---|---|---|---|---|
| Douglas, Haldane and Haldane (1912) | Haemoglobin | CO | $O_2$ | 1/210 |
| Wohl and Glimm (1910) | Amylase | Various carbohydrates | Starch | |
| Quastel and Wooldridge (1927) | Succin-dehydrogenase | Malonic acid | Succinic acid | 1/10 |
| Stedman (1926); Loewi and Navratil (1926) | Choline esterase | Physostigmine | Acetylcholine | |
| Fildes (1940); Woods (1940) | (Unknown enzyme in *streptococci*) | Sulphanil-amide | *p*-Amino-benzoic acid | 300/1 |

* Molar ratios of inhibitor to metabolite when there is 50 per cent inhibition.

lace of the substrate; yet it must be effectively dissimilar hough to fail to carry out the normal functions of the dsorbed substrate. That is, it must either fail to undergo he next chemical reaction in the appropriate metabolic athway, or, if it does undergo it, the product must be nacceptable to an enzyme later in the series.

As evidence that metabolite analogues are acting through *ntagonism by displacement*, there can be cited the case of iotin. This vitamin combines in molecular proportions ith a specific protein, avidin. When biotin sulphone, hich is a physiologically inactive analogue of biotin, is dded to this combination, the biotin is shed from the rotein and can be recovered quantitatively from the olution (Dittmer and du Vigneaud, 1944).

Woolley (1947) has emphasized that competition often ccurs when the metabolite is being built up into a larger nolecule, but never when it is acting as a co-enzyme. For xample, pyrithiamine competes with thiamine which is ot a co-enzyme but a substrate for the enzyme which onverts it to diphosphothiamine, a co-enzyme. It is found nat pyrithiamine has no competitive action against this o-enzyme. Again, pantothenic acid is antagonized by he analogue pantoyl-taurine which prevents it from being uilt up into a larger and more biologically active mole-ule, one with which pantoyl-taurine does not compete McIlwain and Hughes, 1944). Again, *p*-aminobenzoic acid apparently not a co-enzyme, but is an essential raw naterial for the synthesis of folic acid. Sulphonamides ompete against *p*-aminobenzoic acid, but show no bio-gical antagonism towards folic acid (Lampen and Jones, 946).

A word of warning may be necessary that the demonstra-on of competitive antagonism between two chemicals does ot prove that one of them must be a metabolite. Until aminobenzoic acid was isolated from micro-organisms Rubbo and Gillespie, 1940), there were no grounds for suming that it was a metabolite, even though its ability

to antagonize the toxic action of sulphanilamide was well established, and amorphous extracts with very similar properties had been obtained from yeast and bacteria. In certain other cases where competition exists between analogous substances, it has been conceded that neither is a metabolite. For example, the well-known physiological effects of morphine on dogs can be counteracted by allyl-*nor*morphine (Unna, 1943). Again, the yellow dye, trypaflavine, will kill trypanosomes in the mammalian bloodstream, but its action is antagonized by the somewhat similar red dye, parafuchsin. It is quite certain that none of these four substances occurs naturally in mammals. This branch of the study of competition is known as *therapeutic interference* and will be dealt with more fully in Chapter VII.

We should now touch upon some quantitative aspects of competition by metabolite analogues. For each pair of substances there will be a unique *index of inhibition*, which is defined as the ratio of the number of molecules of analogue per molecule of metabolite required to give 50 per cent inhibition. This ratio will vary from species to species, but is always the same for any one species. It obviously is an expression of the relative combining powers of analogue and metabolite for a receptor group in, or on, the cell. It must also include a term for differences in the penetration of the two substances to the site of action, when the latter is not exposed. The amount of inhibition which any analogue can produce therefore depends on two things, firstly, its affinity for the receptor relative to that of the analogue, and, secondly, the relative amounts of analogue and metabolite available at the site of action. A good presentation of the kinetics of these phenomena appears in Work and Work (1948, Chapter IV). Here it will suffice to say that quantitative study of the affinities of competitive inhibitors for enzymes has proved to be very valuable in exploring the affinity of natural substrates for enzymes. Both of these affinities are expressible by constants, known

as Michaelis-Menten constants.* The index of inhibition is obviously the ratio of these two constants.

Some indices of inhibition will be found in Table 1, and it will be noted that two of them are less than unity. This is uncommon, because an analogue rarely has a greater affinity for a natural receptor than has the normal substrate. The larger figure given for sulphanilamide is more typical.

What are the minimal changes in the molecule of a metabolite necessary to turn it into a competitive analogue? Sometimes it is sufficient to rearrange the substituents at an asymmetric carbon atom, if one is present. A few amino-acids respond to this treatment, e.g. the antagonism of *l*-histidine by *d*-histidine, mentioned above. Usually, however, this method is almost ineffective in small molecules, because the space-relationships required for optimal adsorption have been deranged by this treatment: hence a mixture of two optical antipodes usually has the full averaged potency of both, as happens with atropine and adrenaline. The method is more likely to succeed in large molecules, especially if the configuration of only one carbon atom is changed and this carbon is not one involved in adsorption on the receptor. The presence of amino-acids with the 'unnatural configuration' in antibiotic polypeptides probably contributes in this way to their biological activity. *Cis*- and *trans*-isomerides of a large enough molecule should also be antagonistic: the male and female sex hormones of algae (crocetin dimethyl ester) provide an example from nature.

Homologues are sometimes antagonistic, e.g. malonic acid antagonizes succinic acid (see Table 1); glycine antagonizes alanine in some bacteria; β-aminobutyric acid antagonizes β-alanine (in yeast), and methyl folic acid antagonizes folic acid (Chapter II).

For the most part, however, homologues do not make

* The Michaelis-Menten constant ($K_m$) is equal to the concentration of a substrate required to make the appropriate enzyme work at half its maximal velocity.

TECHNOLOGICAL RESEARCH

good competitive analogues; a more radical change is required, and yet it must not be too great. Fluoroacetic acid admirably illustrates this point. This substance occurs in a South African species, *Dichapetalum*, and being highly toxic, has killed off a great deal of cattle in that country. So toxic is this acid that during the last war it was investigated as a possible retaliatory agent in chemical warfare. Now, the toxic action of fluoroacetic acid does not depend on any chemical reactivity on the part of fluorine, because the aliphatic C—F bond is the strongest of all the carbon-halogen bonds. On the other hand, its action depends on the blocking of the metabolic oxidation of various organic acids, particularly citric acid. The effect depends on the small size of the fluorine atom, which enables fluoroacetic acid to masquerade as acetic acid (Barron and Bartlett, 1947). As far as can be seen at the present time, this fluoroacetic acid becomes synthesized by the cell into fluorocitric acid which competes against citric acid in the Krebs' cycle (Liébicq and Peters, 1949; Martius, 1949). Table 2 gives us a quantitative measure of what alterations in the size of groups is compatible with antagonism by metabolite analogues. Evidently the C—Cl distance is too great to permit chloroacetic acid to be adsorbed on acetic dehydrogenase, etc., for this acid is non-toxic. On the other hand, methyl-groups and chlorine atoms are comparable in size (Table 2) and an exchange of chlorine for methyl makes a good competitive analogue out of riboflavine (Kuhn, Weygand and Moller, 1943).

TABLE 2

SOME VAN DER WAALS' RADII

| | |
|---|---|
| H | 1·2 A |
| F | 1·35 |
| Cl | 1·80 |
| $CH_3$ | 2·0 |

Another useful method for obtaining analogues is to make small changes in the atoms constituting a ring. In

otin, it has been found sufficient to replace the sulphur y oxygen, in thiamine (as we have noted before) the ılphur can be replaced by an ethylene group, and the verse change has been successful in the case of phenyl-anine, converting it to thienylalanine which is an anta-onist in micro-organisms.* The substitution of carbon for itrogen has also been successful: guanine is antagonized y benziminazole and riboflavine by its phenazine analogue.

The most generally successful method will now be men-oned, viz. the substitution of one negatively charged roup for another. These may be ionizing groups, as when -COOH is replaced by $-SO_3H$ (successful in the case of icotinic acid) or $-SO_2NH_2$ (as in sulphanilamide); but roups carrying a merely fractional negative charge (i.e. s part of a dipole) are also satisfactory and the ketonic roup has been used to make strongly competitive ana-ogues from nicotinic acid and from *p*-aminobenzoic acid. Vhen designing replacements of this sort, it is important ot to do anything that radically alters the ionization of ny basic group present in the molecule. Nicotinic acid ves no trouble in this respect: the ring nitrogen is already onized (being part of a zwitterion) and hence there is no bjection to replacing $-COOH$ by the stronger acid group $-SO_3H$. On the other hand, it happens that the amino-roup in *p*-aminobenzoic acid is not ionized, so that it is ot admissible to replace $-COOH$ by $-SO_3H$ in this case ecause this would ionize the basic group and hence make ne whole molecule too dissimilar to be a good analogue. ll these considerations have been experimentally verified.

Those who would pursue this question of necessary ructural changes will find further interesting data in Voolley (1947). Many other ways of making metabolite nalogues merely await discovery.

It is all very well to know how to make metabolite nalogues; it is more important, from our point of view,

* It is not possible to replace a sulphur atom by a single carbon atom and ll preserve the flatness of the molecule, an important (steric) consideration.

to know how to make them selective. In those cases where a given metabolite is important in the uneconomic species but not in the economic species, the grounds for selectivity are obvious. It is thus with sulphonamide therapy, because *p*-aminobenzoic acid is very important in microbiological metabolism but apparently unimportant in mammalian metabolism. On the other hand, when a metabolite occurs in all living cells, as thiamine and riboflavine do, one would not at first sight expect any selective employment of analogues to be practicable. However, it does not follow that the analogue would be accumulated equally by both species; some relatively small change can be made in the molecule which will quite alter its distribution coefficient so as to make it (*a*) inaccessible to the economic species or (*b*) selectively adsorbed by the uneconomic one.* By way of example, the vitamin pantothenic acid appears to be an essential metabolite for human beings just as it is for the malarial parasite. However, when a change of $-COOH$ to $-COC_6H_5$ was made (giving phenyl-pantothenone), a very serviceable drug against malaria was obtained, of low toxicity to man.

Curiously enough, a metabolite analogue may be treated as a metabolite by one species and as an antagonist by a related species. For example, dethiobiotin is able to replace biotin as a growth factor for yeast, yet it antagonizes the growth-promoting effect of biotin on *Lactobacillus casei*. It is known why this should be so: the yeast inserts the missing sulphur atom, but the bacterium is unable to do this (Dittmer and du Vigneaud, 1944). Likewise, the fungus *Endomyces*, when made resistant to pyrithiamine by repeated subculture in its presence, was found to owe this resistance to its ability to break down the pyrithiamine and synthesize thiamine from one of the large fragments. A natural metabolite may function in several enzymes, each of which may be optimally blocked by a different inhibitor.

* It is justifiable to make quite a large change to a portion of a molecule known not to lie near to the principal adsorbing groups; an example of such a portion is the pyridine, pyrimidine or thiazole ring in sulphonamide drugs.

example, vitamin K in the human blood-stream can be agonized by dicoumarol, but not by 2:3-dichloro-hthoquinone. On the other hand, vitamin K in gi is powerfully antagonized by this chloro-compound not at all by dicoumarol.

Many cases are known where an analogue can antagonize metabolite in species which require an external source of but not in species which produce it internally. This is case with pyrithiamine, and it looks as though the agonism is exerted on the uptake of the metabolite ough the cell membrane; alternatively, the cell which kes its own metabolite may lack a mechanism by which indiffusible analogue could gain entry to the cell.* ner analogues, such as sulphonamides, benziminazole, 3-dichloronaphthoquinone and phenyl-pantothenone act inst organisms regardless of whether or not they require exogenous source of metabolite.

From what has been said it is obvious that mere know-ge of the metabolites present in an uneconomic species es not enable us instantly to prepare a metabolite logue of practical utility as a selective toxic agent. vertheless, in three cases very valuable drugs have been lved directly from theoretical biochemical considera-ns, and no doubt there will be many more. These drugs phenyl-pantothenone, *p*-aminosalicylic acid and BAL itish Anti-Lewisite).

When it was learnt that pantothenic acid dramatically reased the survival time of malarial parasites *in vitro* ager, 1941), and hence presumably was an important ural metabolite for these organisms, various analogues pantothenic acid were tested as possible antimalarials. e of these, phenyl-pantothenone, was at least as effective man as quinine (Mead *et al.*, 1946). Because of the nmercial availability of older synthetic antimalarials

* Glucose is an example of a substance that cannot penetrate the cell-mbrane by simple diffusion but requires a specific, energy-consuming hanism. For further information on the permeability of natural mem-es, see Davson and Danielli (1943).

('atebrin' and chloroquine), phenyl-pantothenone was not marketed as it showed no particular advantages. This does not in any way detract from the magnitude of the achievement.

The useful antitubercular drug, *p*-aminosalicylic acid, was introduced by Lehmann (1946) following Bernheim's discovery that pathogenic T.B. organisms could use salicylic acid as a substrate. Assuming that salicylic acid was a natural metabolite for these organisms, Lehmann tested 50 of its analogues of which *p*-aminosalicylic acid was most effective.

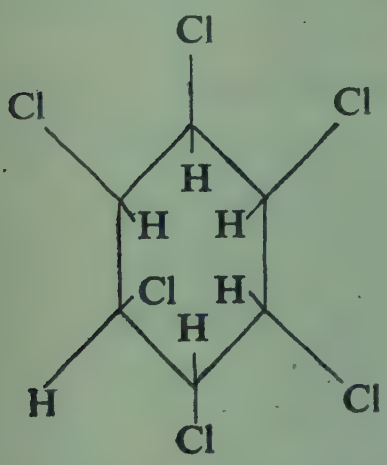
The story of BAL will be told later in this chapter. It is not so much a selectively toxic agent as a selective antidote, being widely used in cases of chronic arsenical and metallic poisoning in man. Several selectively toxic agents in regular use act through their being close analogues of important metabolites, although they were not discovered by planned experiments along these lines. In agriculture, fluoroacetic acid is used as an insecticide and 2 : 3-dichloronaphthoquinone as a fungicide.* Flower-growers and seed-producers water the soil with a 0·1 per cent solution of sodium selenite which is non-toxic to the plants in this concentration. Aphids, red spider and other sucking insects are then poisoned when they swallow the plants' sap, the selenium acting as a metabolite analogue to sulphur in the insect metabolism. Such plants are also poisonous to mammals so that this treatment is not given to crops.

In human medicine, metabolite analogues include the sulphonamides, dicoumarol (which prevents blood-clotting by antagonizing vitamin K), physostigmine and the simpler aromatic urethanes derived from it (these prevent destruction of acetyl-choline by blocking the enzyme which destroys it) and ephedrine (which apparently prevents

* Only the high cost of glutathione prevents it from being used against wilt in tomatoes caused by an analogous polypeptide (lycopersicin) secreted by a fungus, *Fusarium lycopersici*. This remedy was worked out from first principles by Woolley (1946*a*).

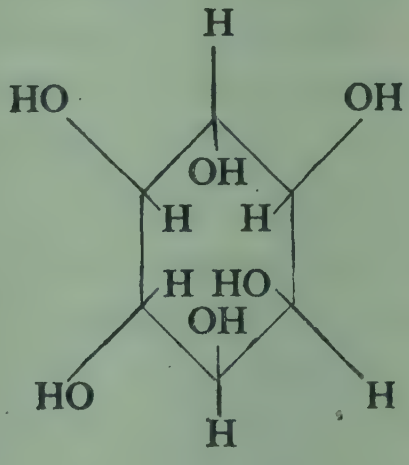
destruction of adrenaline; see Chapter VIII). Actually, the pharmacological possibilities of metabolite analogues have hardly begun to be explored. It is thought highly likely that nature regulates many physiological processes by means of pairs of analogous metabolites, for example the polyene sex-hormones of algae (Kuhn, 1940), the porphyrins (Granick and Gilder, 1945), the mammalian sex hormones (Woolley, 1946*b*) and possibly the sympathetic-stimulants, adrenaline and *nor*adrenaline.

The case of the powerful insecticide hexachloro*cyclo*hexane ('gammexane') deserves special treatment. It was suggested by Slade (1945) that gammexane (VII) is the *meso*-form of the nine possible geometrical isomerides of this substance and that it probably destroys insects by antagonizing natural inositol (VIII), which is the *meso*-form of hexahydroxy*cyclo*hexane and is known to be of fairly general importance in metabolism.




Gammexane

(VII)




Inositol

(VIII)

Kirkwood and Phillips (1946) showed that a yeast which required an external source of inositol was not seriously poisoned by *alpha*-, *beta*- or *delta*-isomerides of gammexane, but that gammexane itself was distinctly toxic. On the addition of inositol, most of the toxic effect of gammexane was counteracted, but the slightly toxic effects

of the other isomerides were unchanged. This seemed to support Slade's hypothesis, but no comparable results were obtainable in insects, although carefully sought. Moreover, the X-ray diffraction study of gammexane has shown that it is not *meso* in structure (van Vloten *et al.*, 1948) and hence it is not an exact analogue of *meso*-inositol. Thus, after all, gammexane may not be acting by analogue antagonism.*

In concluding this section on metabolite analogues, it may be recalled that the principle involved has already led to some useful selectively toxic agents and is certain to lead to others. In addition, it has been immensely helpful in the unravelling of metabolic processes, thus increasing our understanding of cell biochemistry which in turn must lead to the discovery of selectively toxic agents, including various types other than metabolite analogues.

### (*b*) *The covalent bond in relation to selective toxicity: the arsenicals and mercurials*

A link between this section and the last is seen in the simplest alcohols. Ethanol (ordinary alcohol) can be considered as a metabolite analogue, the metabolite being water. Like water, the alcohols have strong hydrogen-bonding properties and there is little doubt that alcohol penetrates the body by ways ordinarily reserved for water. For example, both alcohol and water pass readily through the stomach wall, although the stomach is an organ for storage and is designed to allow no part of a digested meal, except water, to pass into the blood-stream. The symptoms which follow the absorption of alcohol provide evidence that, although it may masquerade as water up to a point, there is a stage beyond which the tissues are not deceived. The intoxication produced by alcohol appears to be a

* Gammexane interferes with mitosis in onions and this toxic effect can be reversed by *meso*-inositol, as can the interference caused by colchicine. It is thought that the cellular component with which both gammexane and colchicine interfere is not *meso*-inositol itself, but a substance to which it gives rise and the formation of which is prevented by these mitotic poisons (Chargaff, Stewart and Magasanik, 1948).

ypical Ferguson effect and it is not surprising that the ıigher alcohols act in ever-lower doses.

What is surprising, at first sight, is that methanol is ıore toxic than ethanol. Inspection of the formula of ıethanol, however, shows that it differs from its higher ıomologues in not being a primary alcohol; indeed it has ɔne hydrogen atom too many, and it must be classed as ın alcohol of 'zero order'. It is much more readily oxidized ɔ the corresponding acid than are the primary alcohols, ınd many of the toxic symptoms of methanol are attributed ɔ the accumulation of formic acid in sensitive tissues, such ıs the retina of the eye. Hence, the essential difference ɔetween the physiological action of methanol and ethanol ɛsides in the greater ease with which the former can take ɔart in a reaction involving the making and breaking of ɔovalent bonds and leading to a toxic product.*

Many covalent bonds are made and broken in the normal ıetabolic processes of the cell and also in disposing of ıany foreign materials, accidentally introduced (cf. Williams, 1947). However, there is no reason to believe ɬhat covalent bonds play an important part in the mode ɔf action of many selectively toxic agents. The strength ɔf the covalent link is almost always so great† that equili-ɔrium processes at or below 37° C. could not occur. Yet ve know that such equilibria are typical of the action of ıany toxic agents. For example, it is common to find ɬhat the progress of the toxic effects can, if taken in time, ɔe reversed by the application of an antidote. In many ɔases dialysis or simple dilution with water is a sufficient ıntidote.

One well-established case where selectively toxic action depends on covalent bond formation is that of the

* This product (formic acid) is not toxic in all parts of the body, e.g. the ɔlood-stream. Its toxicity is connected with its being in tissues to which it is ıormally denied access (because of unfavourable distribution coefficients) but ɔ which methanol has ready access. Fortunately ethanol is more strongly ıdsorbed than methanol, so that the inhalation (or consumption) of a given ımount of methanol in methylated spirit is not so harmful as would otherwise ɔe expected (Røe, 1943; Zatman, 1946).

† See Chapter II.

arsenicals and mercurials. Here the abnormally weak As—S and Hg—S bonds are responsible. Before dealing more thoroughly with this case, it is interesting to review the not very numerous examples where drugs are believed to be degraded in the body before the medicinally desirable substance is produced.

Some arsenical and mercurial drugs depend for their action on a change in the state of oxidation (see below).

'Prontosil' (IX) does not act until it is reduced to sulphanilamide in the blood-stream.*

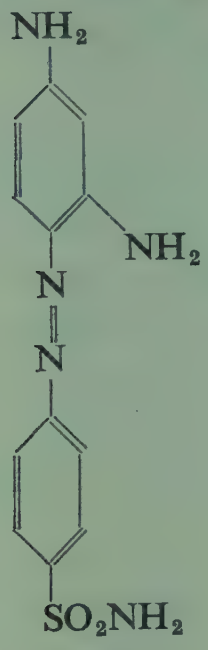
The antimalarial, 'paludrine', is altered in the blood to a more active material as is shown by the blood being more plasmocidal than the amount of unaltered drug found in it (Hawking and Perry, 1948). The same phenomenon occurs with another antimalarial, 'plasmoquine' (Blanchard and Schmidt, 1946; Schönhöfer, 1942).

It would not be easy to quote many more examples of this kind. Perhaps the iodinated quinolines that are used as amoebicides act by the liberation of nascent iodine following the local oxidation of the C—I bond. The liberation of iodine may also explain the strong fungicidal action of iodinated hydrocarbons in which the carbon-iodine bond is weakened by electron-attracting substituents. For example, tri-iodonitroethylene is more than 500 times as effective as iodoform (tri-iodomethane) (Muirhead, 1949; McGowan, 1949).

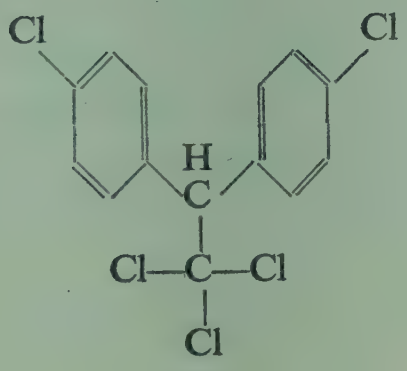
An attempt to explain the mode of action of the powerful insecticide DDT (dichloro-diphenyl-trichloromethane) (X) along these lines was made by Martin and Wain (1944). These authors noted that DDT readily loses hydrochloric acid on treatment with alkalis, giving dichloro-diphenyl-dichloro-ethylene (XI) which is non-toxic to insects. However, it seems unlikely that the liberation of small amounts of hydrochloric acid in, presumably, well-buffered tissues can account for the violent convulsive action of this substance.

* No such breaking of covalent bonds is involved in the action of other sulphonamides commonly used in medicine such as sulphadiazine.

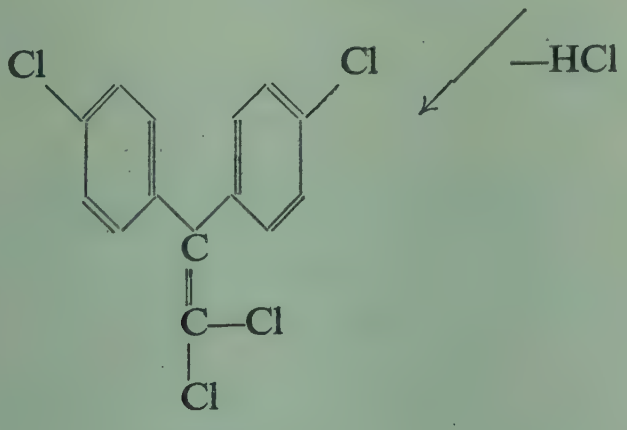
hat the amount of acid would be small, will be realized om the fact that as little as $10^{-12}$ gm. of DDT per sq. n. is sufficient to kill flies settling on it. The Martin-Vain hypothesis has been criticized by Busvine (1945,



(IX)
'Prontosil'



(X)
DDT

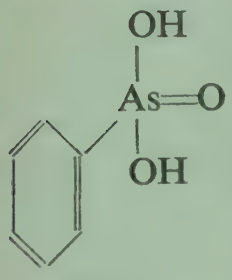


(XI)

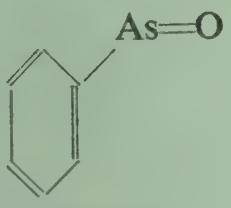
946), who showed that the toxicity to lice and bugs of a eries of analogues of DDT was not proportional to the ase of hydrolysis. However that may be, the overriding act is that chlorine-free analogues of DDT, such as : 1-dianisyl*neo*pentane, retain 25 to 50 per cent of their

activity (Brown and Rogers, 1950). Thus no grounds remain for trying to connect the action of DDT with the breaking of covalent bonds. Some of the selectivity of DDT for insects may be explained by the observation that chitin absorbs it very strongly (e.g. up to 2 per cent from dilute solution), whereas cellulose and keratin do not (Lord, 1948).

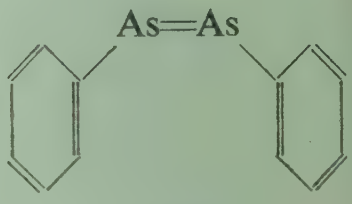
The organic arsenicals, which are derivatives of benzene, are available at three different levels of oxidation (XII*a*, *b* and *c*). Firstly, there are the arsonic acids (XII*a*) in which the arsenic is pentavalent. Next come the arsenoxides (XII*b*) in which the arsenic is trivalent. Lastly, there are the arsenobenzenes (XII*c*) in which the arsenic is again trivalent but at a lower level of oxidation, so that the arsenobenzenes rapidly oxidize in the air to arsenoxides.




(XII*a*)




(XII*b*)



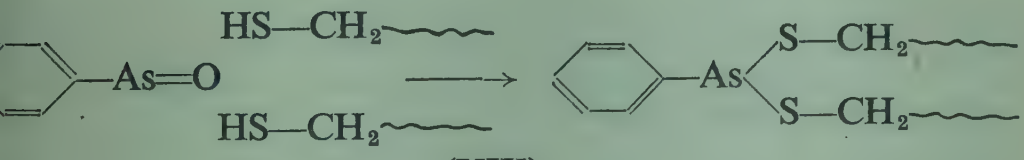

(XII*c*)

The pentavalent arsenicals are physiologically inactive as such but penetrate rapidly to all parts of the body. They are mainly excreted unchanged but, as Ehrlich showed, many tissues can reduce small amounts of these substances to the corresponding arsenoxides, i.e. to the level at which arsenic is therapeutically active. In medicine, advantage is taken of the good penetrating properties of pentavalent arsenicals to generate small amounts of a therapeutically active arsenical in a site which it could not normally reach. In neuro-syphilis and trypanosomiasis, for example, pentavalent arsenicals such as tryparsamide are used because they penetrate to the seat of the infection in the central nervous system, where trivalent arsenicals cannot readily

ter. In the earlier stages of syphilis, where the infection quite accessible, trivalent arsenicals are preferred.

The mode of action of arsenicals was worked out be-veen 1920 and 1925 by Voegtlin in the United States ublic Health Service. Historically, this was the first time at the mode of action of a selectively toxic agent was xplained in chemical terms. A photograph of Voegtlin is own overleaf.

Voegtlin and his co-workers (1920, 1923, 1924, 1925) owed that the activity of arsenobenzenes was due to eir oxidation to arsenoxides which then reacted with tally important thiol groups in the parasite. The chemical action in aqueous solution between arsenoxides and iols was known in Ehrlich's time. It occurs instantly, most quantitatively and in the cold. This reaction is own in (XIII), and it is remarkable in that the covalent s—S bonds which are formed can readily be broken, in e absence of a slight excess of thiols, by simple dilution.




(XIII)

Although Ehrlich (1909) was well aware that pentavalent rsenicals did not act in the body until reduced to arsen-xides, it never occurred to him that arsenobenzenes acted nly after oxidation to arsenoxides and that, as Voegtlin owed, the arsenoxide level is the only one at which rsenicals are therapeutically active. In Fig. 1, it can be en that 'mapharside' (XIV) brings about a dramatic wering of the trypanosome population in the rat's blood-ream within half an hour of administration, but 'salvar-n' (XV, arsphenamine), which is the corresponding rsenobenzene, requires over 5 hours to accomplish the me result. It would seem that arsphenamine has to ange to a more active substance before it can act.

Voegtlin showed that if salvarsan is shaken with air for a few minutes, it is converted to its arsenoxide (mapharside) which then exerts the typical rapid action. The same result is obtained if the arsphenamine is incubated with oxidizing tissues, such as fresh liver.

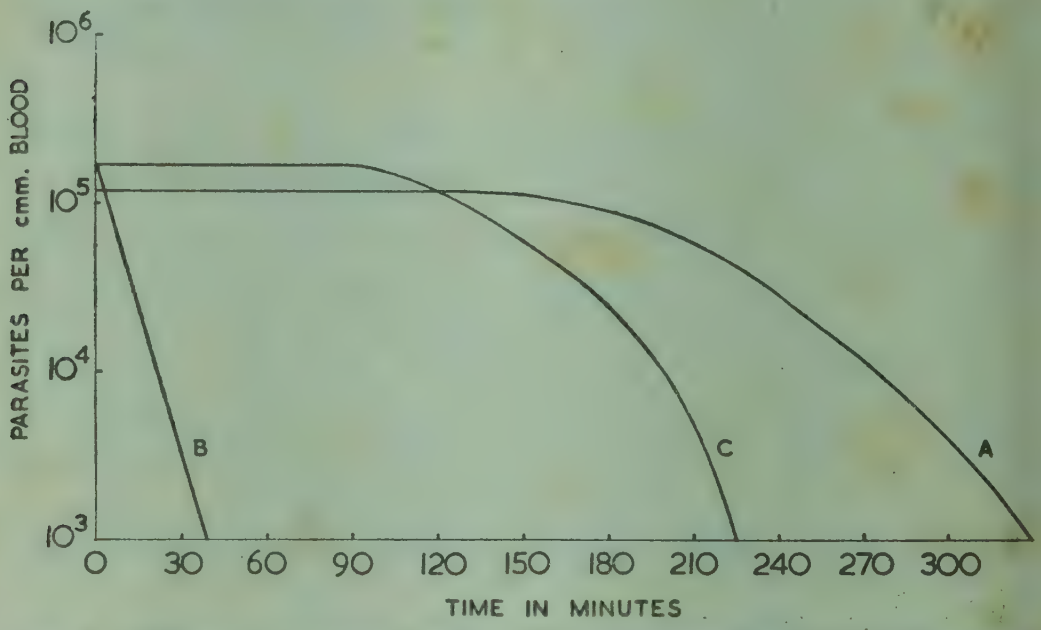
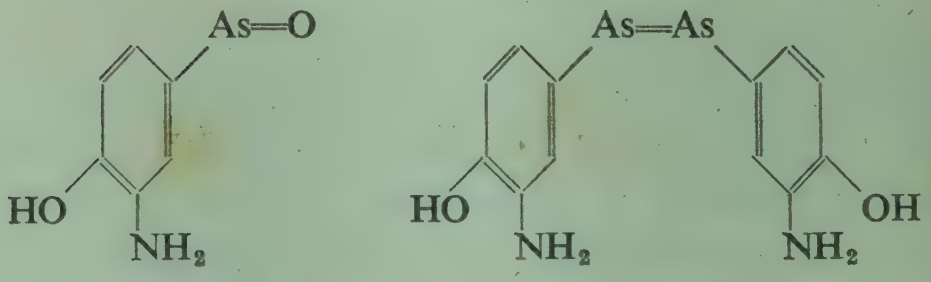



Fig. 1—The parasiticidal effect, in trypanosome infected rats of (A) arsphenamine, (B) 'mapharside', and (C) 'mapharside' plus reduced glutathione.
(*redrawn from Voegtlin, 1925*)




'Mapharside' (XIV)

'Salvarsan' (XV)

Fig. 1 also illustrates Voegtlin's other important discovery, namely that the action of arsenoxides is antagonized by substances containing thiol groups such as glutathione, cysteine, sodium thioglycollate or sodium thiolactate. This effect is not durable if the amount of

1. Carl Voegtlin

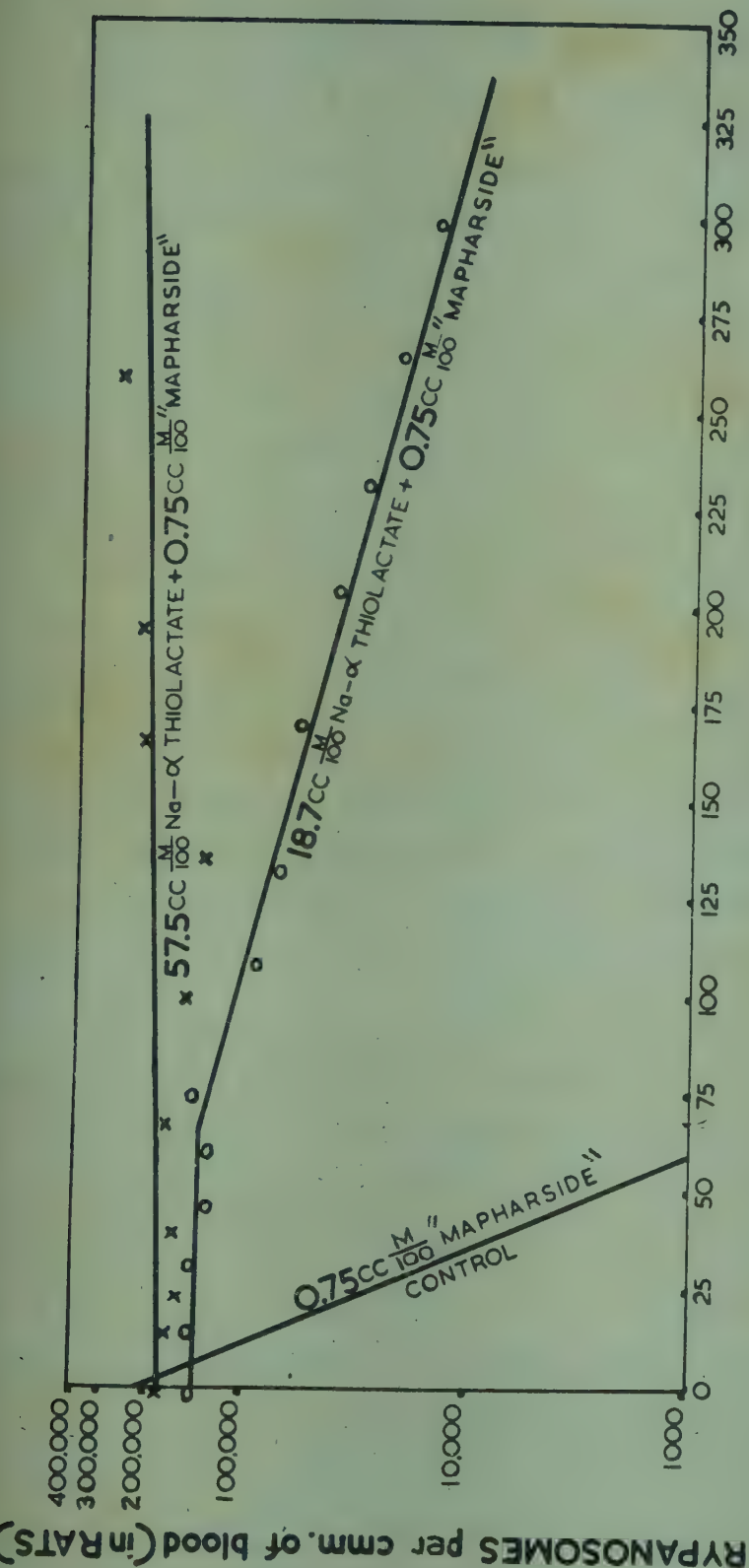



Fig. 2—The inhibition by sodium thiolactate of the trypanocidal effect of 'mapharside' in infected rats.
(*redrawn from Voegtlin*)

thiol is small, because, as was pointed out above, the complex can hydrolyse back to its original components. With larger amounts of thiols, the effect is prolonged because of the working of the mass-action law (see Fig. 2).

These results led Voegtlin to investigate an untried suggestion, made by Ehrlich (1909), namely that arsenoxides act by combining with sulphur in the parasite. Voegtlin showed that trypanosomes are abundantly provided with thiol-groups, because they give the nitroprusside reaction and other tests for $-SH$. The importance of thiol-groups in cell-activity of all kinds had already been underlined by the work of Gowland Hopkins from 1917 onwards, and at the present day we know some forty enzymes that are inactivated if their thiol-groups are chemically blocked. Such an enzyme is succinic dehydrogenase (VI). We have already discussed how it can be blocked by malonic acid because this is an analogue of its normal metabolite, succinic acid. However, it can also be blocked by allowing it to react with an organic arsenical. This combines with the $-SH$ group and gives a blocking effect which cannot be reversed by succinic acid (as can the inhibition caused by malonic acid), but it can be reversed by thiols such as glutathione.*

Thus, it is easy to see how the toxic action of organic arsenicals on trypanosomes can be exerted by the blocking of essential thiol-groups in the parasites. That this is truly the mode of action of these drugs is supported by the fact that no other groups with which arsenoxides could react have been found in the parasites, whereas the abundant thiol-groups present could not fail to react with the arsenoxides available from the blood-stream. It should be pointed out here that this is essentially a reversible reaction and that parasites that have been treated with several lethal doses of arsenic can be saved if subsequently treated with many equivalents of a thiol-compound. It is quite obvious

* It is interesting to note that succinic acid, by 'roofing over' the thiol-group of succinic dehydrogenase, can completely protect this enzyme from the blocking action of arsenicals.

that, in an infected animal, an arsenoxide drug is partitioned between the thiol-compounds of the host and those of the parasite and is in a state of dynamic equilibrium.

This theory of Voegtlin's is very satisfying, although it leaves questions yet to be solved. To what are the vital —SH groups of the parasite attached? Voegtlin at first thought that they formed part of a small molecule such as glutathione; later, the —SH groups of proteins were thought responsible (Rosenthal and Voegtlin, 1930) and it is now generally believed that a respiratory enzyme having an essential thiol-group is intimately involved. How do organic arsenicals injure the parasite without harm to the host, although they combine with a chemical group which is vital to both parasite and host? Inorganic and aliphatic arsenicals are no less toxic to trypanosomes than the aromatic arsenicals, but with the exception of a few aromatic arsenicals, all arsenic-containing substances are too toxic to mammals to be used as selectively toxic agents. Clearly, the secret of the selectivity lies in the aromatic (benzene) ring and the substituents which it carries. This conclusion has been confirmed by drug-resistance studies (see also Chapter VII) which seem to point to selective adsorption. There is room for much more work here.

Voegtlin's studies have been extended by his colleagues Rosenthal (1932) and Eagle (1939), and it is now known that arsenical drugs act on spirochaetes by the same mechanism as on trypanosomes. No matter at what level of oxidation the drug may be, it has first to be converted to the arsenoxide level and it then combines with essential thiol-groups in the parasite.

Because Ehrlich had, mistakenly, taught that arsenoxides were too toxic to be used as drugs, syphilis was treated for a quarter of a century with unnecessarily large doses of arsenic, in the form of salvarsan and similar arsenobenzenes. A small (but sometimes dangerously large) portion of those massive doses was converted to the potent arsenoxides in the body; the greater part of it engaged in

side-reactions which were unpleasant, and sometimes dangerous, for the patient. Eventually, two Americans, Tatum and Cooper (1932, 1934) showed that the arsenoxide corresponding to salvarsan was a safer drug than salvarsan itself because, although it was more toxic to the host, it was even more toxic to the parasite and hence smaller doses were effective. This substance (*m*-amino-*p*-hydroxyphenylarsenoxide (XIV), known in England as 'mapharside' and in America and Australia as 'mapharsen') has largely replaced the arsenobenzenes in the United States, and may well do so here. This drug has made it possible to cure syphilis with small, safe doses of arsenic.

More recently, it was suggested that mercurials, such as mercuric chloride and phenyl mercuric nitrate, exert their antiseptic action on bacteria by combining with essential —SH groups (Fildes, 1940*a*). The bacteria appear to be quite dead, but are easily revived by treatment with a thiol such as thioglycollic acid or even hydrogen sulphide (Chick, 1908).

This work of Fildes created an apparently paradoxical situation: if arsenicals act on trypanosomes and spirochaetes by inactivation of essential thiol-groups (as Voegtlin showed) and if mercurials act on common bacteria by inactivation of essential thiol-groups also (as Fildes demonstrated), then it would be a logical necessity for arsenicals to be powerful antibacterials *in vitro*. There was at that time little in the literature to suggest that this was so, indeed there were a number of definite statements that organic arsenicals were not antibacterial. To resolve this paradox, my colleagues and I determined to test arsenical compounds at each of the three levels of oxidation, against a representative series of bacteria. The results are given in Table 3. It will be seen that arsenicals can be strong antibacterial agents, like mercurials, provided that they are used at the right level of oxidation (Albert, Falk and Rubbo, 1944). As expected, the active level of oxidation is the arsenoxide level (the organisms are

## TABLE 3

### THE EFFECT OF ORGANIC ARSENICALS ON BACTERIA

Greatest dilutions completely inhibiting visible growth in 48 hours at 37°C (medium: Peptone broth, containing 10% serum. pH 7·2.)

| *Substance* | *Organism* | | | | |
|---|---|---|---|---|---|
| | *Cl. welchii* | *Strept. haem A* | *Staph. aureus* | *B. coli* | *Proteus* |
| Acetarsol (*m*-acetamido-*p*-hydroxyphenyl-arsonic acid) . . . . . | * | * | * | * | * |
| 'Mapharside' (*m*-amino-*p*-hydroxyphenyl-arsenoxide) . . . . . | 1:160,000 | 1:80,000 | 1:160,000 | 1:10,000 | 1:10,000 |
| Neoarsphenamine (*m*-amino-*p*-hydroxy-arsenobenzene-*N*-methylenesulphoxyl-ate) . . . . . | 1:10,000 | 1:10,000 | 1:10,000 | * | * |
| Mapharside in 0·1% thioglycollate broth | 1:5,000 | 1:10,000 | * | * | * |
| Mercuric chloride (for comparison) . | 1:40,000 | 1:160,000 | 1:40,000 | 1:80,000 | 1:80,000 |

* Signifies not inhibitory, even at a concentration of 1 : 5,000.

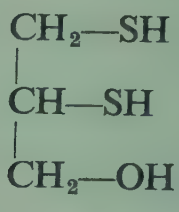
Albert, Falk and Rubbo (1944).

apparently not able to convert much of the material from other levels to this level). Also as expected, the antibacterial action of the arsenoxide is neutralized by sodium thioglycollate.

The toxic action of the arsenicals used in chemical warfare is also connected with their property of forming covalent bonds with —SH groups. Lewisite ($ClCH:CH.AsCl_2$), the most vesicant member of this series, produces lesions in man that are not reversed or even averted by any mono-thiol. The work done by Peters and his colleagues at Oxford from 1923 onwards led to the discovery that lewisite injures the skin and exerts widespread toxicity by blocking —SH groups of an enzyme system, viz. pyruvate oxidase (Peters, 1936, 1948). Further research at Oxford during the last war showed that injuries caused by lewisite could be completely reversed by dithiols in which two —SH groups were fairly close together (Peters, Stocken and Thompson, 1945). The best of these dithiols (2 : 3-dimercaptopropanol (XVI) also known as BAL and dimercaprol) has since proved to be an excellent antidote for arsenical poisoning accidentally arising in the treatment of syphilis and also for acute and chronic poisoning arising from various metallic compounds. The effect of this antidote is the best proof that arsenic attacks essential —SH groups.

Other substances than mercurials and arsenicals can inactivate —SH groups, e.g. oxidizing agents (such as iodine), alkylating agents (particularly iodoacetamide), arylating agents (particularly quinones and quinonimines) and aldehydes. During the Second World War much work was done in Oxford, Cambridge (Dixon and Needham, 1946), Liège (Bacq, 1946) and in the U.S.A. to show that several non-arsenical chemical warfare agents exert their toxic action in this way. The idea was extended to a number of naturally occurring substances by Cavallito and Haskell (1945). Among such substances have been numbered clavacin, penicillic acid, hexenolactone (which is

found in malt, yeast and orange peel), pyocyanin and the principles of the flowering plants, *Arctium minus* and *Allium sativum*, because the toxic action can be reversed by cysteine, glutathione and other thiols. However, these substances are not *selectively* toxic, and hence do not merit comparison with the aromatic arsenical drugs. The toxic effect of penicillin on bacteria can be reversed by particular thiols (viz. cysteine and its methyl ester and β-dimethylaminoethylthiol), but these must be used in considerable excess and in concentrated solutions. The more usual thiols, thioglycollic acid and glutathione, have no effect. Hence penicillin does not seem likely to act merely by exerting a direct blocking effect on vital —SH groups. Further information on the mode of action of penicillin will be presented in Chapter VII.



BAL
(XVI)

## *CHAPTER FOUR*

# THE ROLE OF IONIZATION IN SELECTIVE TOXICITY. THE ACRIDINES AND OTHER KATIONIC ANTIBACTERIALS. THE CARBOXYLIC ACIDS AND PHENOLS

### (*a*) *The role of ionization in selective toxicity*

Among the various factors which are responsible for the action of selectively toxic agents, ionization phenomena must be accorded a very important place. Many selectively toxic agents are capable of ionization and, of these, many ionize to different degrees at different pH values. Conversely, at a given pH value (such as the physiologically important pH of 7), members of a series of selectively toxic agents, even though of almost identical chemical structure, will usually be found to be ionized to quite different degrees.

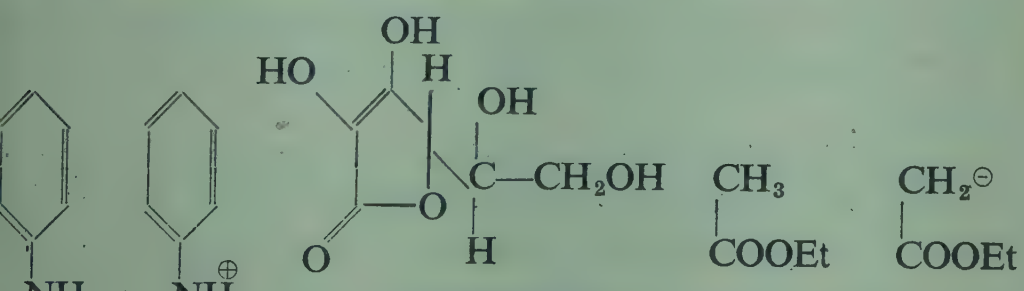
These facts would be unimportant if both an ion and its corresponding neutral molecule had the same biological effects. But in all cases that have been investigated, ions and neutral molecules behave differently. These differences are quantitatively significant, sometimes partaking of an all-or-nothing character. Hence, it would be useful for us to devote a whole chapter to the study of ionization phenomena.*

The role of ionization in selective toxicity can best be understood if we examine the role of ionization in chemistry and biochemistry. There are at least three circumstances in which ions and molecules behave differently. These are *chemical reactivity*, *adsorption at surfaces* and the *penetration of membranes*.

*Chemical reactivity.* All organic chemists know that the molecule (I) of aniline becomes nitrated in the *ortho*- and *para*-positions, whereas its ion (II) becomes nitrated

* The following account assumes at least a nodding acquaintance with the subject of ionization. Kolthoff and Laitinen's (1941) book on pH and electrochemistry would be useful collateral reading for the more advanced reader.

only in the *meta*-position. By controlling the acidity of the nitration mixture, the proportions of the isomerides can be controlled. An example of greater biochemical interest is ascorbic acid (III) which is readily oxidized by air when present as the di-anion, whereas the mono-anion and the neutral molecule (both of which can be made the principal species present, by a change in pH) are quite stable (Weissberger and LuValle, 1944). Many molecules have acidic or basic groups so weak that they do not ionize appreciably at ordinary pH values; nevertheless it often happens that the few ions that they do produce have such extreme chemical instability that a useful chemical reaction can be brought about. In such cases, the decomposition of a few ions permits the formation of more, according to the law of mass action. For example, ethyl acetate (IV) is quite stable, but the anion (V), formed only in traces in strongly alkaline solutions,* rapidly reacts with a molecule of ethyl acetate to give ethyl acetoacetate; further anions are then formed and, after a few hours, a large proportion of the ester has been converted (Watson, 1941). Conversely, strongly acid solutions often produce small amounts of highly active kations from stable molecules, as Hammett (1935) showed when examining the pH region between 0 and minus 9. For example, the active nitrating agent in a mixture of nitric and sulphuric acids is the nitronium kation ($-NO_2^{\oplus}$); similarly the highly reactive sucronium kation is the first step in the hydrolysis of cane-sugar by acids.




Ascorbic acid

(I) (II) (III) (IV) (V)

* These must be non-aqueous to prevent hydrolysis.

*Adsorption at surfaces.* There are two kinds of adsorption, the indiscriminate and the specific. Indiscriminate adsorption is shown by those substances which G. S. Hartley classified as *amphipathic*, i.e. having a water-attracting end-group attached to a comparatively large water-repelling residue. Ordinary soap is an example of this class of substances. It will be recalled that water molecules are extensively hydrogen-bonded to one another and that substances dissolve in water only by virtue of their ability competitively to break some of these bonds and form new hydrogen bonds with water molecules. Amphipathic molecules, dissolved in water, are in a state of uneasy equilibrium, because the hydrophobic portion is constantly being squeezed out by the water molecules in their endeavour to unite with one another in its vicinity. If such a hydrophobic portion is squeezed out of solution, its hydrophilic end-group must of necessity go with it; conversely, the more strongly the end-group is bound to water molecules, the more resistant the amphipathic molecule, as a whole, can be to this squeezing-out process. This tendency of water to squeeze out amphipathic substances gives rise to the phenomenon of indiscriminate adsorption because such substances tend to be deposited on any surface that presents itself, regardless of its chemical nature. Soap, for instance, is thus caused to accumulate at the air-water interface of a vessel in which it is stored, and it simultaneously accumulates at the glass-water interface. If objects are immersed in the soapy solution, soap will be found to accumulate on them also, largely regardless of their chemical nature. This is typical indiscriminate adsorption.

In such indiscriminate adsorption, neutral molecules tend to be adsorbed more strongly than ions, e.g. oleic acid is more strongly adsorbed than (sodium) oleate. The reason is that an ion is more strongly hydrated than the corresponding neutral molecule so that the latter has the greater tendency to undergo expulsion.

Specific adsorption is a phenomenon often shown by hydrophilic substances whereby they tend to leave water to accumulate on a surface that has a chemically complementary character. One of the commonest kinds of complementarity is that an anion becomes attracted to a positively charged portion of a surface or, conversely, a kation is attracted by an anionic portion of a surface. In such cases an ion is obviously adsorbed much more strongly than the corresponding neutral molecule. Many ingenious applications of specific ionic adsorption have been worked out in mineral flotation (cf. Rogers, Sutherland, Wark and Wark, 1946). Later, we shall be discussing some important examples of this phenomenon among selectively toxic agents.

*Penetration of membranes.* Biologists are in agreement that every cell is surrounded by a semi-permeable, invisible membrane which is about four molecules in thickness. According to Davson and Danielli (1943), this usually consists of two monolayers of lipoids surrounded on either side by a monolayer of protein. Such a membrane is strongly charged and its interior is difficultly accessible to ions. In fact, these authors devote an entire chapter (Chapter XIV) to showing that, for any given substance, the neutral molecule penetrates very much better than the corresponding ion* (see also Höber, 1945, Chapters X and XI). The difficulties in the way of the penetration of ions are (*a*) their relatively greater size, due to hydration, and (*b*) their charge which is either similar to the portion of protein surface which they approach, resulting in repulsion, or is opposite, resulting in fixation by adsorption. One is irresistibly reminded of the ease of penetration of atoms by neutrons, which are uncharged, whereas electrons and protons, because of their charge, penetrate only with the greatest difficulty.

In view of these three categories of physico-chemical

* The addition of lipophilic groups to ions increases their ability to penetrate natural membranes (non-ionic end first). This may explain the value of the chlorine atom in 'atebrin'.

differences between ions and molecules, it is not surprising that some selectively toxic agents are active only in the non-ionized state and others are most potent in the ionized state. Later we shall meet one series which achieves maximal activity only when both states are simultaneously present. However, before we consider actual instances, we should first refresh our memories with the necessary quantitative aspects of ionization phenomena.

The most important quantitative consideration to bear in mind is that at any given pH value, the proportion of ions to neutral molecules depends only on the $pK_a$ of the substance under examination. Thus, if one were determining the toxicity of salicylic acid to a fungus, it would not matter whether one worked with salicylic acid itself or with sodium salicylate, because the buffered medium would convert both of these substances to a mixture having the same ratio of ion to neutral molecule. Given the pH of the buffer and the $pK_a$ of salicylic acid, this ratio can be read off (as a percentage) from Table 2.

For the benefit of students, it should be pointed out that $pK_a$ values are numerals used to classify acids and bases according to strength.* The $pK_a$ is numerically equal to the pH at which a substance is half neutralized (i.e. half ionized and half non-ionized). Actually, the $pK_a$ is the negative logarithm of the ionization constant, $K_a$, which is defined by the following equations for acids and bases respectively:

$$K_a = \frac{[A^{\ominus}]\,[H^{\oplus}]}{[AH]} \text{ and } \frac{[B]\,[H^{\oplus}]}{[BH^{\oplus}]}, \qquad \ldots (1)$$

where $[A^{\ominus}]$ stands for concentration of anions, $[BH^{\oplus}]$ concentration of kations, [AH] concentration of non-ionized acid (i.e. neutral molecule), etc.

* The majority of acids and bases ionize to different degrees at a given dilution and this is an expression of their relative strengths. Salts, on the other hand, are completely ionized. However, solutions of the salts of *weak* acids or bases will be partly hydrolysed to the feebly ionized acids or bases.

It is pleasant to see that the tendency to write $K_b$ values for bases (instead of $K_a$ values) is passing out of use. The strengths of both acids and bases are most conveniently described on the $K_a$ scale, just as acidity and alkalinity are both measured by pH without invoking pOH.

TABLE 1

RELATIVE STRENGTHS OF SOME COMMON ACIDS AND BASES

| *Acids* | *pKa* | *Bases* | *pKa* |
|---|---|---|---|
| Hydrochloric acid, Sulphuric acid | * | Sodium hydroxide, Guanidine | * |
| Oxalic acid, Phosphoric acid | 2 | | 12 |
| | 3 | Ethylamine | 11 |
| | 4 | | 10 |
| Acetic acid | 5 | Ammonia | 9 |
| Carbonic acid | 6 | Quinine, Strychnine | 8 |
| | 7 | | 7 |
| | 8 | | 6 |
| Hydrocyanic acid, Boric acid | 9 | Aniline, Pyridine | 5 |
| Phenol | 10 | | 4 |
| | 11 | | 3 |
| | 12 | | 2 |
| Glucose | 13 | *p*-Nitroaniline | 1 |

* Too strong to have a $pK_a$ value.

Table 1 presents, in a form suitable for memorizing, a selection of $pK_a$ values for common acids and bases. It will be seen that a few acids, like hydrochloric acid, are too strong to be classified on the $pK_a$ system, but somewhat less strong acids, e.g. oxalic and phosphoric acids, are known to have the $pK_a$ value of 2. Typical aliphatic and aromatic acids, like acetic and benzoic acids, have the $pK_a$ of 5, and carbonic acid is ten times weaker* with its $pK_a$ of 6. Acids with $pK_a$ values less than 7 scarcely redden litmus,

* Ten times, because $pK_a$ values are logarithmic.

e.g. hydrocyanic and boric acids with the $pK_a$'s of 9, but phenol ($pK_a = 10$) does not even taste acidic. We may not always realize when eating glucose that it, too, is an acid, and although its $pK_a$ is 13, many weaker acids are known.

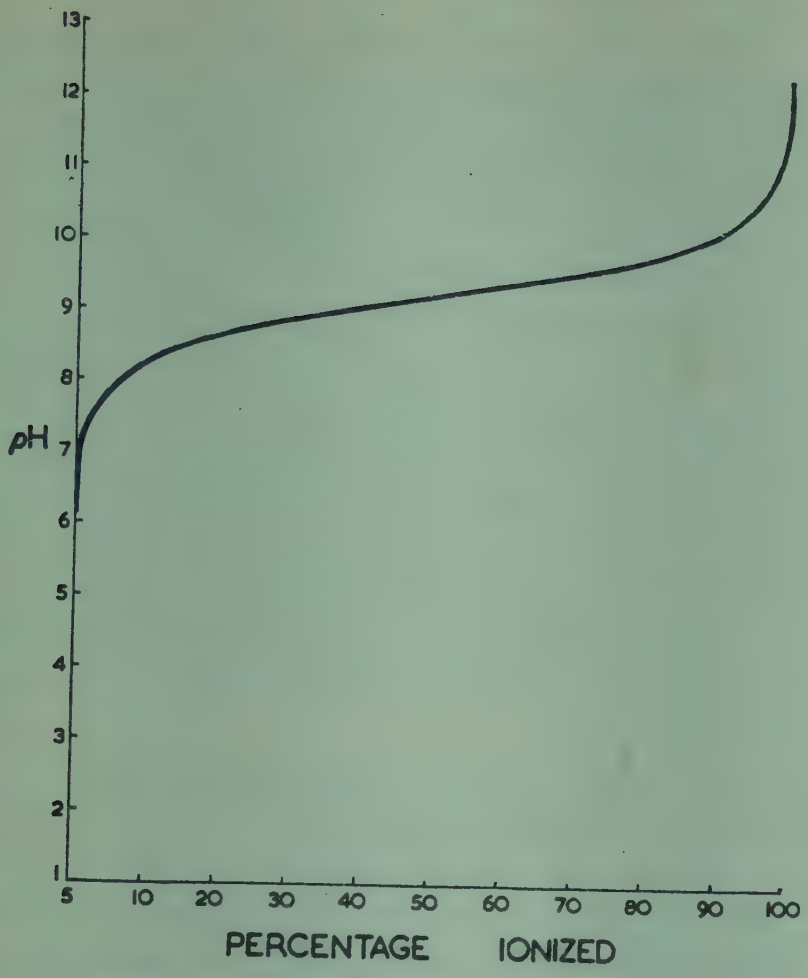
The classification of bases in Table 1 follows similar lines, starting with alkalis like sodium hydroxide which are too strong to have a $pK_a$ value and passing through the aliphatic bases (typified by ethylamine, $pK_a = 11$), ammonia ($pK_a = 9$), the majority of alkaloids ($pK_a = 8$) to the aromatic and heteroaromatic bases (which scarcely turn litmus paper blue and are typified by aniline and pyridine, with $pK_a$ values of 5) and continuing, through *p*-nitroaniline ($pK_a = 1$) to some extraordinarily weak bases (such as anthraquinone* ($pK_a = -8$).

The degree of ionization of any base in any aqueous solution can be calculated from the following equation, provided that two things are known, the pH of the solution and the $pK_a$ of the substance:

$$\text{Per cent ionized} = \frac{100}{1 + \text{antilog}\,(pH - pK_a)}. \quad \ldots (2)$$

This equation shows that the degree of ionization varies with pH, but that it does not bear a straight line relationship to the extent of the change. On the contrary, a sigmoid curve is formed, as in Fig. 1. It will be seen from this figure that a small change in pH can make a large change in ionization, particularly if the pH of the solution is numerically close to the $pK_a$ of the substance investigated. This is readily seen from Table 2. By way of example, if one were working at pH 7 with a nitrophenol of $pK_a = 7$, half of the substance would be in the ionized state. If the pH were allowed to rise to 8, the phenol would be nearly all ionized, but if it fell to pH 6, the phenol would be mainly in the non-ionized condition.

* Measured by Hammett (1935) when working at pH −9.




ig. 1—Typical curve obtained in the potentiometric titration of an acid (boric acid, $pK_a = 9{\cdot}21$ at 20°).

## TABLE 2*

ALCULATION OF THE EXTENT OF IONIZATION, GIVEN $pK_a$ AND pH

| $pK_a - pH$ | *Per cent ionized (if anion)* | *Per cent ionized (if kation)* |
|---|---|---|
| −4 | 99·99 | 0·01 |
| −3 | 99·94 | 0·10 |
| −2 | 99·01 | 0·99 |
| −1 | 90·91 | 9·09 |
| 0 | 50·00 | 50·00 |
| 1 | 9·09 | 90·91 |
| 2 | 0·99 | 99·01 |
| 3 | 0·10 | 99·94 |
| 4 | 0·01 | 99·99 |

An extended form of this table will be found as Appendix II at the end his book (p. 206).

At this stage, it may well be asked, 'How can the degree of ionization matter where the action of a toxic agent is concerned? Surely poorly ionizing substances produce at least a few ions and as these are removed, fresh ones are generated from the neutral molecules according to the Mass Action Law, as in equation (1)?'

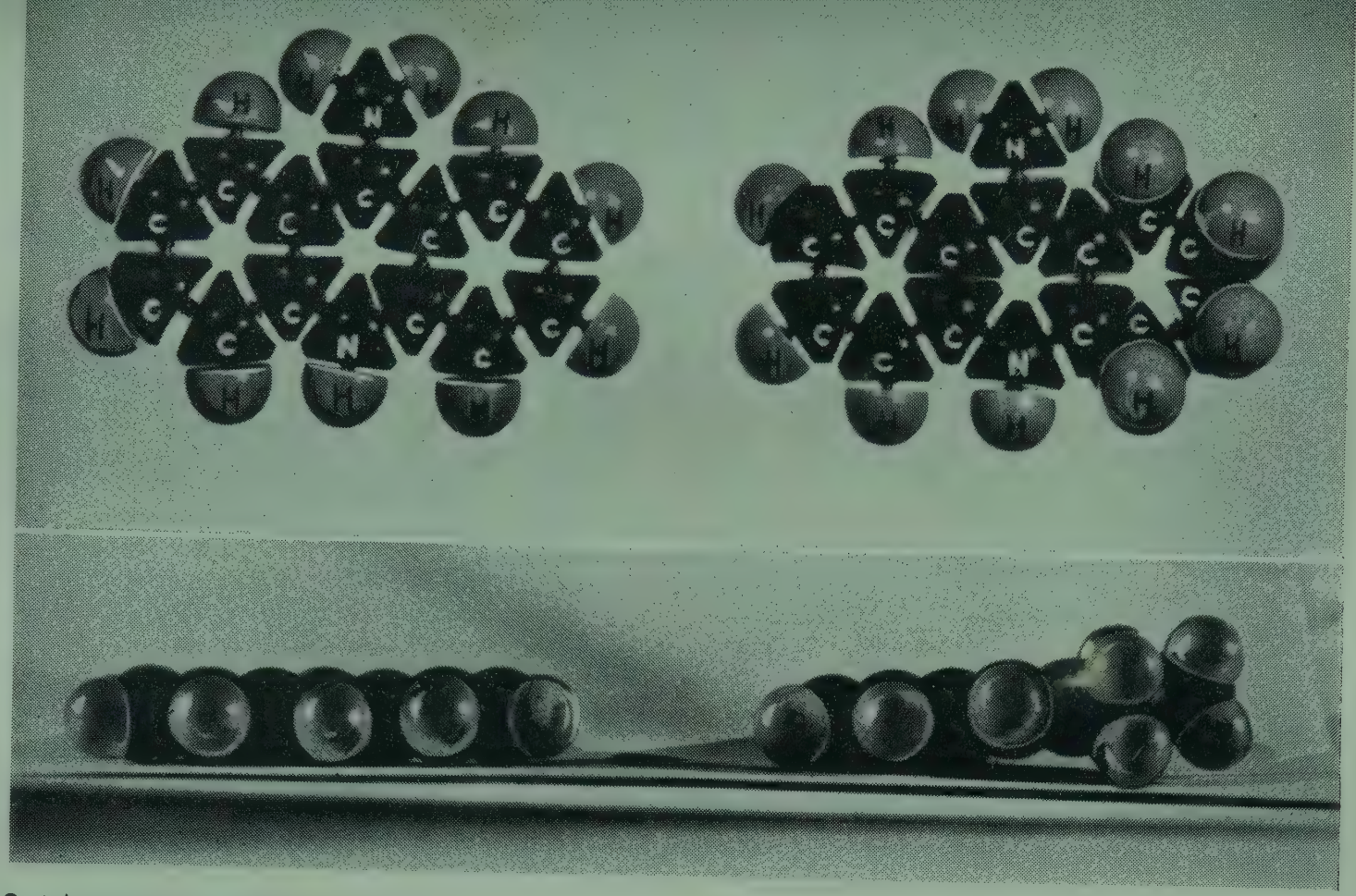
This objection is reasonable, but the type of action envisaged has not yet been found to occur in nature. On the contrary, all selectively toxic ions contradict this assumption by ceasing to be active at dilutions where they are still present in solution. For example, crystal violet is bacteriostatic to *B. coli* at a dilution of 1 in 10,000, but not at 1 in 20,000, although both solutions are intensely violet. The violet colour denotes the present of the kations, which are the active form of this antibacterial (the neutral molecule is colourless).

Equations (3) and (4) help to explain this phenomenon. Equation (3) shows that the magnitude of $K_I$ (the ionization constant) governs the proportion of kation ($BH^+$) to neutral molecule (B) at any given hydrogen ion concentration [$H^+$]. Equation (4) similarly deals with the magnitude of $K_S$ which is the stability constant of the complex (ABH) formed by combination of the kation ($BH^+$) of the drug with the vulnerable anionic group [A−] of the bacterium. This complex cannot be an ionized one, otherwise the stability will be vanishingly small. The drug and receptor-anion will be united by an ionic bond, it is true, but another type of bond must exist as well for $K_S$ to reach any useful magnitude.

$$K_I = \frac{[B]\,[H^+]}{[BH^+]} \quad \text{. . (Ionization constant)} \qquad \ldots (3)$$

$$K_S = \frac{[ABH]}{[A^-]\,[BH^+]} \quad \text{. . (Stability constant)} \qquad \ldots (4)$$

If, as is usually the case, $K_I$ and $K_S$ are of comparable magnitudes, then any deficiency of kations in the solution



2. Accurate space models of the ions of 5-aminoacridine (left-hand figures) and tetrahydro-5-aminoacridine (right-hand figures) (*see p. 96*)

will be replenished from the drug-bacterium complex (ABH), and not only from the non-ionized drug (B). Under these circumstances the ionization constant of the drug becomes the limiting factor governing its potential usefulness: not only must ions be present, but they must be present abundantly.

Returning to crystal violet, we may note that *Streptococcus pyogenes* is inhibited by a dilution of 1 in 320,000 but not by a dilution of 1 in 640,000. A dilution of 1 in 2,000,000 will still kill *Staphylococcus aureus*. Hence there are different $K_S$ values for different kinds of cells, and this makes a selectively toxic action possible.

We are now in a good position to examine actual cases where biological action has been found to depend upon the degree of ionization.

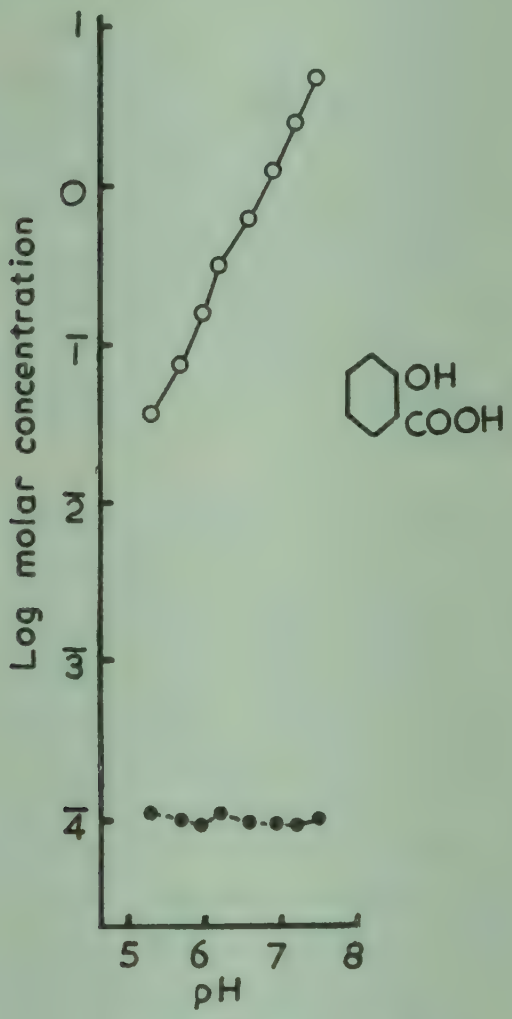
(*b*) *Cases where ionization is disadvantageous to toxic action*

The inhibition by salicylic acid ($pK_a = 3{\cdot}0$) of cell division in an echinoderm egg is shown in Fig. 2. It is seen from the upper curve that salicylic acid is more active at pH 5 than at any other pH tested. At pH 5, a larger proportion of the salicylic acid is non-ionized than at any higher pH. However, when the inhibitory concentration of non-ionized salicylic acid is plotted (lower curve) it is found not to change, regardless of the pH. It is obvious that, in this case, the neutral molecule is the only toxic form of the substance. Hence, the more acid the solution, the more economical the use of salicylic acid will be, because of the higher concentration of neutral molecules that will be present. This tendency for weak acids to be most biologically active when non-ionized was first noticed by Vermast (1921) who investigated the antibacterial action of benzoic acid at various pH values.

Similarly, it has been shown that all members of a series of 30 barbiturates enter both eggs and larvae of the sea-urchin *Arbacia* exclusively as neutral molecules, and the resulting depression of cell-division and respiration,

respectively, is also due to neutral molecules exclusively (Clowes, Keltch and Krahl, 1940).

The tendency for neutral molecules to be more effective than ions is shown in Table 3, which deals with the




*Data of Smith*, 1925

Fig. 2—The effect of pH on the concentrations of salicylic acid required to stop the cell division of *Echinarachnius parva*.

Upper curve: total drug (=ions+neutral molecules).
Lower curve: Neutral molecules.

action of various narcotics on the worm *Arenicola* (Clowes and Keltch, 1931). It can be seen that the effect of non-electrolytes, such as chloroform, is independent of pH. On the other hand, weak bases, such as cocaine, are most

TABLE 3

THE CONNEXION BETWEEN IONIZATION AND THE NARCOSIS OF *ARENICOLA*

(Minimal Anaesthetic Doses, in g. per 100 ml. of sea-water, rendering this worm immobile after 5 minutes)

| | pH 7·0 | pH 8·0 | pH 9·0 |
|---|---|---|---|
| NON-ELECTROLYTES | | | |
| *iso*Propyl alcohol | 2·5 | 2·5 | 2·5 |
| *iso*Amyl alcohol | 0·1 | 0·1 | 0·1 |
| Chloroform | 0·012 | 0·012 | 0·025 |
| Chlorbutol | 0·025 | 0·025 | 0·025 |
| WEAK BASES ($pK_a$ = *c.* 8·5) | | | |
| Cocaine | 0·01 | 0·005 | 0·0025 |
| Procaine | 0·002 | 0·001 | 0·0005 |
| Butyn | 0·001 | 0·0002 | 0·0002 |
| BARBITURIC ACIDS ($pK_a$ = *c.* 8·0) | | | |
| *iso*Amyl, ethyl- ('amytal') | 0·006 | 0·025 | 0·05 |
| Propylmethylcarbinyl, ethyl ('nembutal') | 0·003 | 0·006 | 0·012 |
| Diethylcarbinyl, ethyl- | 0·006 | 0·012 | 0·05 |
| *n*-Amyl, ethyl- | 0·006 | 0·012 | 0·05 |

Clowes and Keltch (1931).

effective in the more alkaline solutions where they are least ionized. Also the weak acids (four isomeric barbiturates) are least effective in the more alkaline solutions where they are most ionized. Thus it is evident that the narcotic action* depends upon neutral molecules rather than ions. However, in this case it is possible to calculate from the ionization constants that the ions are making a *small* contribution to the toxic action. Such examples of combined action will be further dealt with under (*d*).

These studies were simplified by the fact of the pH change having, of itself, no effect on the test organism. Less fortunate were some workers who investigated the

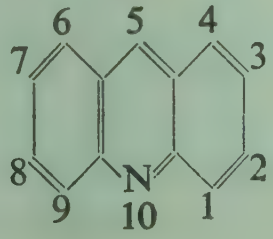
* This narcotic action is readily reversed in each case by washing the worms with more sea-water. It is, most likely, a pure Ferguson effect (see Chapter II).

action of quinine derivatives on bacteria. They believed they had shown that the neutral molecules were more active than the ions because these drugs became more effective as the alkalinity rose (Michaelis and Dernby, 1922). Unfortunately they had overlooked the ionizing effect of alkali on the bacterial receptors (see section (*c*)).

(*c*) *Cases where ionization is advantageous to toxic action*

The classic example of a positive correlation between ionization and biological activity is found in the acridine antibacterials. Actually Stearn and Stearn (1924) had suggested that the order of increasing antiseptic action among the triphenylmethane dyes was the same as the order of increasing basic strength. Unfortunately, this hypothesis could not be checked because determination of the ionization constants presented considerable difficulties. These difficulties, which have recently been overcome (Goldacre and Phillips, 1949), hinge on the rather unusual phenomenon of a pseudo-base taking part in the equilibrium, which is not reached for several hours. It was found that there is an indication of the sort of correlation suggested by the Stearns. However, the position is complicated by the varying times taken for equilibrium by the various pseudo-bases.

In the acridine series, pseudo-bases are rare and avoidable. Hundreds of examples can be found where equilibria are instantaneous: in other words, the ionization process is *purely* ionic. Accordingly, my colleagues in Australia and I (1939–1947) decided to seek a correlation between ionization and antibacterial activity in this series. Typical results are given in Table 4.




(VI)

## TABLE 4

DEPENDENCE OF BACTERIOSTASIS ON IONIZATION IN THE ACRIDINE SERIES

(For further examples and other bacteria, see Appendix I, p. 200)

| Acridine | *Min. bacteriostatic concentration for* Strept. pyog. *after* 48 *hours' incubation at* 37°. (*Medium*: 10 *per cent serum broth*; *pH*=7·3) | *Per cent Ionized* (*pH* 7·3; 37° *C.*) |
|---|---|---|
| -Amino- . . | 1 in 80,000 | 73 |
| -Amino- . . | 160,000 | 100 |
| :5-Diamino- . | 80,000 | 98 |
| :5-Diamino- . . | 160,000 | 100 |
| :7-Diamino- . . | 160,000 | 76 |
| :8-Diamino- . . | 160,000 | 99 |
| -Amino-1-methyl- . | 320,000 | 100 |
| -Amino-2-methyl- . | 160,000 | 100 |
| -Amino-3-methyl- . | 160,000 | 100 |
| -Amino-4-methyl- . | 320,000 | 100 |
| -Amino-1-chloro- . | 80,000 | 83 |
| -Amino-2-chloro- . | 160,000 | 96 |
| -Amino-3-chloro- . | 160,000 | 94 |
| -Amino-4-chloro- . | 160,000 | 86 |
| Amino- . . | 5,000 | <1 |
| Amino- . . | 10,000 | 2 |
| Amino- . . | 10,000 | 2 |
| 9-Diamino- . . | <5,000 | <1 |
| 7-Diamino- . . | 20,000 | 3 |
| Amino-5-methyl- . | 20,000 | 3 |
| Amino-1-methyl- . | 20,000 | 1 |
| Amino-9-methyl- . | <5,000 | <1 |
| Amino-8-chloro- . | <5;000 | <1 |
| Amino-5-chloro- . | <5,000 | 11 |
| Amino-7-chloro- . | 40,000 | 20 |
| Amino-8-chloro- . | 40,000 | 33 |

Albert, Rubbo, Goldacre, Davey and Stone (1945).

There are only five possible mono-aminoacridines, two of which are well ionized at pH 7, whereas the remainder are poorly ionized. It can be seen from Table 4 that the two isomerides which are well ionized have a powerful antibacterial action, whereas the three that are poorly ionized have only a feeble action.

The same correlation is seen in the remaining parts of Table 4. We may compare the well-ionized diamino-acridines with the poorly ionized diaminoacridines, the well-ionized methyl-aminoacridines with the poorly ionized methyl-aminoacridines and the well-ionized chloro-amino-acridines with their poorly ionized isomerides. In all cases ionization has brought about a vast increase in activity: eightfold to sixteenfold in most cases. Plainly, structure is relatively unimportant in this series, except in so far as it influences ionization. The conclusions were reinforced by the examination of one hundred acridines, variously substituted, using a representative range of bacterial species (Albert, Rubbo, Goldacre, Davey and Stone, 1945; Albert and Goldacre, 1948). These results, which furnish one of the most clearly established correlations in the whole field of selective toxicity, will be found in Appendix I to this volume. As the influence of ionic resonance and of inductive effects in the acridine series have been worked out (Albert and Goldacre, 1946), it is now possible to predict the $pK_a$ of an acridine in advance of making it, thus saving time and materials.

Thus, ionization has been shown to be by far the most important limiting factor governing the antibacterial action of acridines; but this ionization must be kationic in character. By inserting acidic groups into the nucleus of a strongly basic acridine, it is easy to make a substance which is zwitterionic, and hence no longer kationic. Such a substance is 5-aminoacridine-3-carboxylic acid (Table 5). It is seen that this has lost its antibacterial action, but it regains it upon esterification which restores it to the kationic condition. By inserting acidic groups into the

TABLE 5

IMPORTANCE OF KATIONIC IONIZATION FOR BACTERIOSTASIS IN THE ACRIDINE SERIES

| *Substance* | *Min. bacteriostatic concentration for* Strept. pyog. *after 48 hours' incubation at 37°. (Medium: 10 per cent serum broth; pH=7·3.)* | *Per cent Ionized (pH 7·3)* | | | |
|---|---|---|---|---|---|
| | | *Kation* | *Anion* | *Zwitter-ion* | *Neutral molecule* |
| 5-Aminoacridine . . . | 1 in 160,000 | 100 | 0 | 0 | 0 |
| 5-Aminoacridine-3-carboxylic acid . . . . . | <5,000 | 0 | 0·2 | 99·8 | 0 |
| Methyl ester of above . . | 160,000 | 94 | 0 | 0 | 6 |
| Acridine . . . . | 5,000 | 0·3 | 0 | 0 | 99·7 |
| Acridine-5-carboxylic acid . | <5,000 | 0 | 99·3 | 0·7 | 0 |
| Methyl ester of above . . | <5,000 | 0 | 0 | 0 | 100 |

Albert, Rubbo, Goldacre, Davey and Stone (1945).

nucleus of a weakly basic acridine, an anionic substance is formed. Such a substance is acridine-5-carboxylic acid (Table 5). It is seen that this substance, like its ester and like acridine itself (none of which are kationic), has no appreciable antibacterial action.

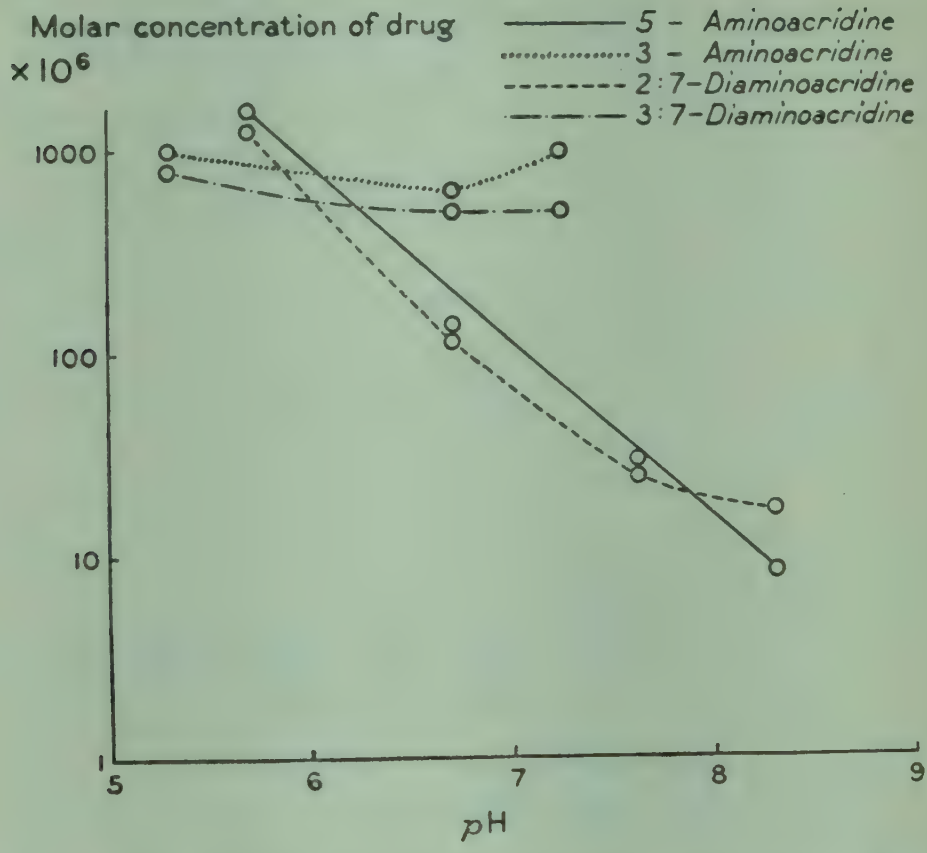
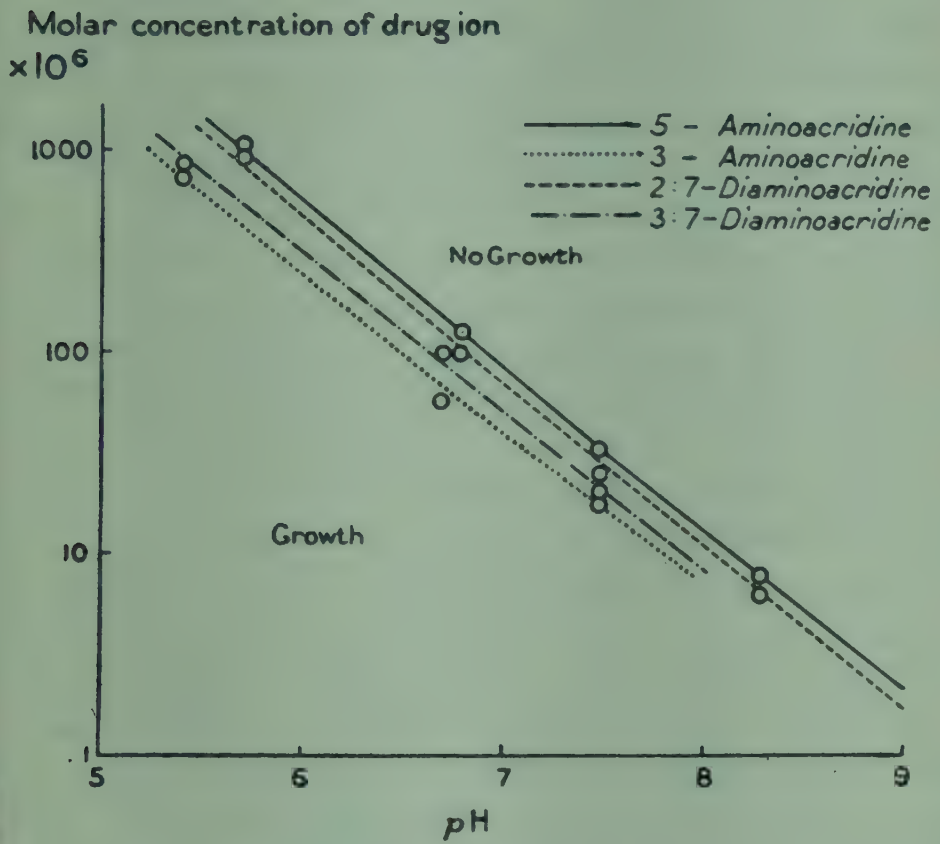



Fig. 3—Competition between hydrogen ions and acridines (ions+molecules).
Organism: *B. coli.*

Much more has been learnt about the mode of action of acridines by using a test organism that withstands both acid and alkaline media (*B. coli*). In Fig. 3, the logarithms of the minimal bacteriostatic concentrations of four acridines have been plotted against a range of pH values from 5·5 to 8·5. One of these acridines is a strong enough base to be ionized throughout this pH range, the others are not. It will be seen that the result is a rather disorderly set of

urves. However, when the amount of actual kation nstead of the total amount of substance) is plotted on ne ordinate, an orderly set of curves is obtained, as shown n Fig. 4. These curves consist of parallel straight lines, nd they lead to a number of interesting conclusions.




g. 4—Competition between hydrogen ions and acridine ions. Organism: *B. coli*.

Firstly, it is evident that the most important factor overning bacteriostasis at any pH is the amount of drug esent as kation (this depends on both the pH of the edium and the $pK_a$ of the drug; see Table 2) and not e total amount of drug (kations+neutral molecules). condly, the slope of the curve shows that there is a direct mpetition between acridine kations and hydrogen ions. ne simplest interpretation of the mode of action of ridine kations is that they compete with hydrogen ions

for a vitally important anionic group on the bacterium. This group is blocked by combination with the acridine kation and is thereby prevented from discharging its normal metabolic functions.*

This anionic group appears, from Fig. 4, to have a $pK_a$ of 9 or higher: thus it could be the hydroxy-group in a tyrosine residue of a protein or in the purine and pyrimidine constituents of nucleic acid. That kations, of the same molecular weight as those under discussion, can liberate hydrogen ions from bacteria is plainly shown in Table 6.

The third conclusion to be derived from Fig. 4 is that clinically a smaller amount of any aminoacridine could be effectively antibacterial in wounds if these were prevented from becoming acid. Some very interesting work along these lines was carried out by the Australian Army during the recent war and it was recommended that sodium bicarbonate lavage should precede treatment with 5-aminoacridine hydrochloride.† The good results of this therapy established it as a valuable measure for the prevention and treatment of sepsis in wounds. It is true that sulphonamides and penicillin might also be used for this purpose, but there is a tendency to avoid them because of the danger to subsequent parenteral therapy if drug-resistance or drug-sensitivity sets in, as not infrequently happens. Drug-resistance to 5-aminoacridine is unknown.

There are two reasons for believing that these acridine antibacterials exert their action on the outside of the bacteria, (i) examples which are entirely ionized are at least as active as those which are partly ionized (contrast with sulphonamides, see (*d*) below), and (ii) the addition of aliphatic side-chains, which would assist penetration of the cell-membrane, actually reduces the antibacterial action (Albert, Rubbo, Goldacre, Davey and Stone, 1945). It is known that cells have many vital enzymes on their exterior surface; many of these are concerned with assimilation.

* For some more complex but less likely alternatives, see Albert, 1951.

† This useful aminoacridine has recently become official in the British Pharmacopoeia, under the name 'Aminacrine' Hydrochloride.

## TABLE 6

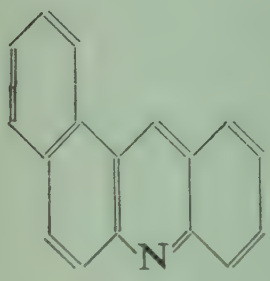
LIBERATION OF HYDROGEN IONS FROM *BACILLUS BELLUS*, CAUSED BY ADSORPTION OF CRYSTAL VIOLET KATIONS

| (1) *pH of bacterial suspension* | (2) *pH of crystal violet solution* | (3) *pH of mixture of 1 and 2* | *Fall in pH caused by adsorption* |
|---|---|---|---|
| 8·5 | 8·5 | 6·6 | 1·9 |
| 6·5 | 6·3 | 4·8 | 1·7 |
| 5·5 | 5·5 | 3·8 | 1·7 |
| 5·0 | 5·1 | 3·5 | 1·6 |
| 4·1 | 4·2 | 3·0 | 1·1 |

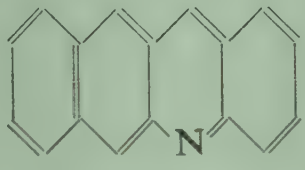
McCalla (1941).

The relationship between kationic ionization and antibacterial activity holds not only for the acridine series but for at least seven other series, without further qualification.

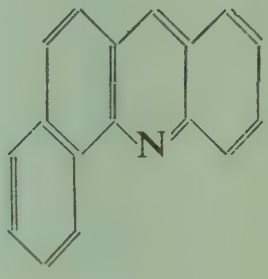
These are the three series of benzacridines (VII, VIII and IX), the three series of benzquinolines (X, XI and XII) and the phenanthridines (XIII) (Albert, Rubbo and Burvill, 1949).



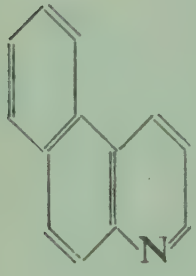

(VII)




(VIII)

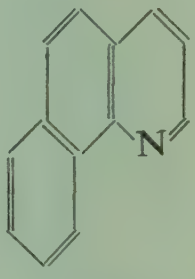



(IX)

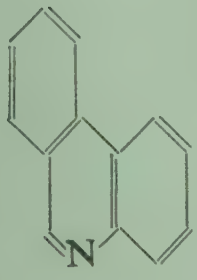



(X)


(XI)




(XII)




(XIII)

When the antibacterial action of the 24 acridine compounds in Table 4 is plotted against percentage ionization, a curve is obtained which reveals the underlying relationship (Fig. 5). Similar curves have been obtained

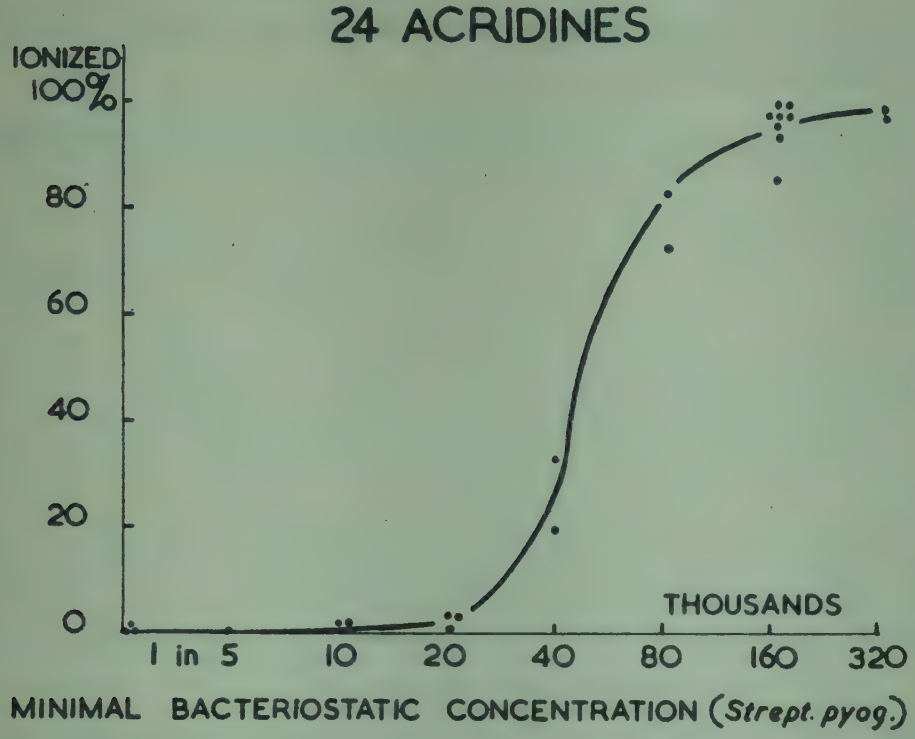
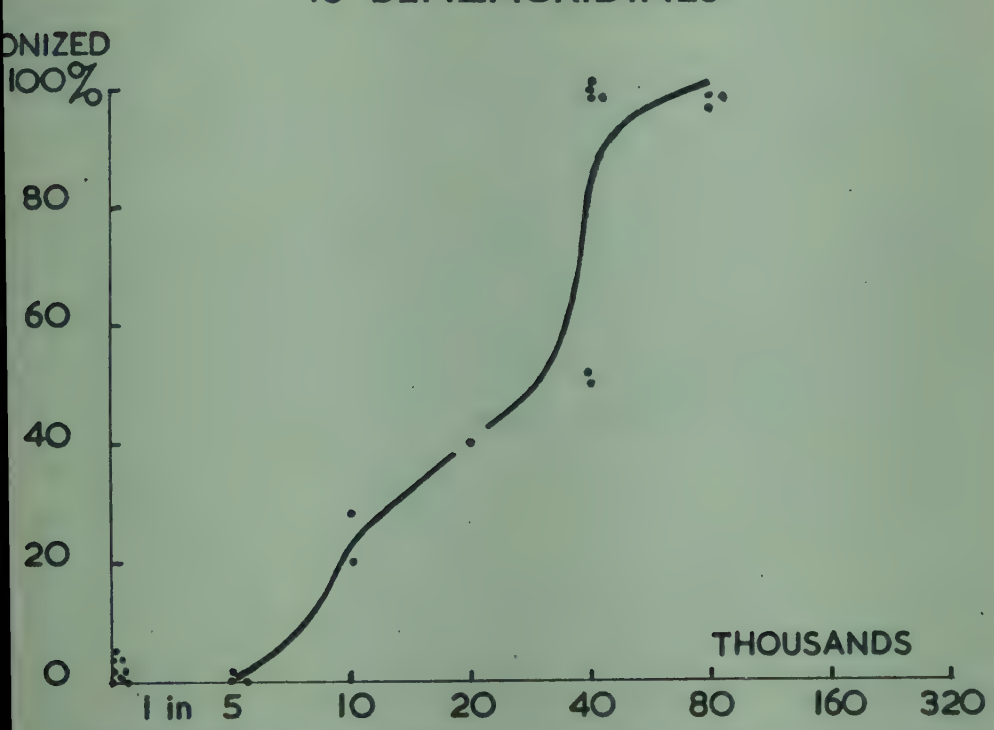



Fig. 5—Effect of ionization on the antibacterial action of acridines.




g. 6—Effect of ionization on the antibacterial action of benzacridines.

for the benzacridines (Fig. 6) and the benzquinolines and phenanthridines (Fig. 7).

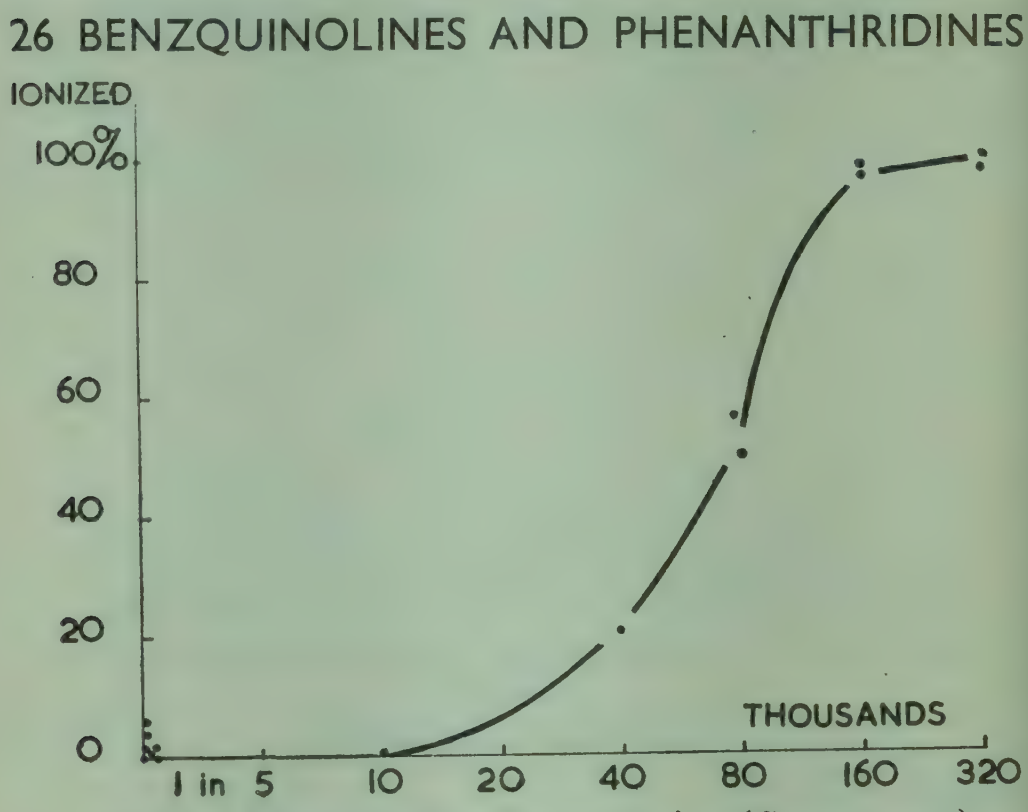
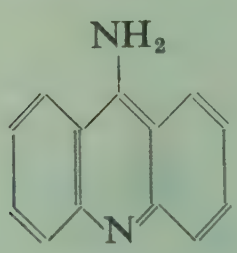
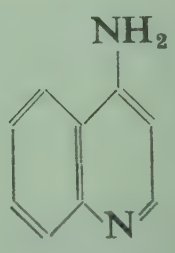



Fig. 7—Effect of ionization on the antibacterial action of benzquinolines and phenanthridines.

When we turn to the lower benzologues of acridine, the first thing we notice is their lack of antibacterial powers. This is shown in the sequence (XIV, XV, XVI), the figures in brackets referring to the minimal bacteriostatic concentration against *Strept. pyog.*, as described in Table 4.

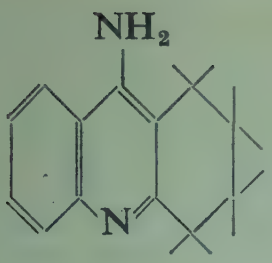



(XIV)
5-Aminoacridine
(1:160,000)

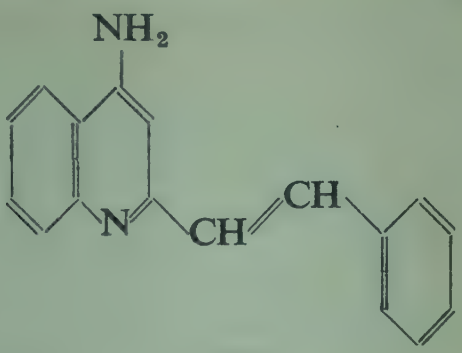



(XV)
4-Aminoquinoline
(<1:5,000)


(XVI)
4-Aminopyridine
(<1:5,000)




(XVII)
5-Aminotetrahydroacridine
(1:5,000)




(XVIII)
4-Amino-2-styryl-quinoline
(1:80,000)

The lack of antibacterial properties in 4-aminoquinoline XV) and 4-aminopyridine (XVI) was found to be corre-ted with a lack of a sufficient area of flatness in these olecules. If an envelope (as shown in Fig. 8) is awn around various heterocyclic nuclei, an area of only sq. A is obtained for quinoline, whereas acridine, enanthridine and the benzquinolines have an area of sq. A. All the heterocyclic nuclei discussed so far are rfectly flat, because they are conjugated throughout. owever, when the highly antibacterial 5-aminoacridine IV) is hydrogenated in one ring, giving (XVII), only sq. A of flat surface remain, because hydrogenated rings e always three-dimensionally voluminous. Simultane-sly with this change in flat area, the antibacterial activity s almost completely vanished. It should, therefore, be ssible to create highly antibacterial quinolines and ridines by the insertion of groups which will increase total area of flatness of the molecule. It is known from ray diffraction studies that stilbene is a perfectly flat lecule (Robertson and Woodward, 1937),* hence it was ught that the addition of a styryl group to 4-amino-noline (giving XVIII) would produce a highly anti-terial compound. This indeed proved to be the case, l the addition of two styryl groups to 4-aminopyridine s similarly successful (Albert, Rubbo and Burvill, 1949).

* Diphenyl, on the contrary, is not flat (Karle and Brockway, 1944).

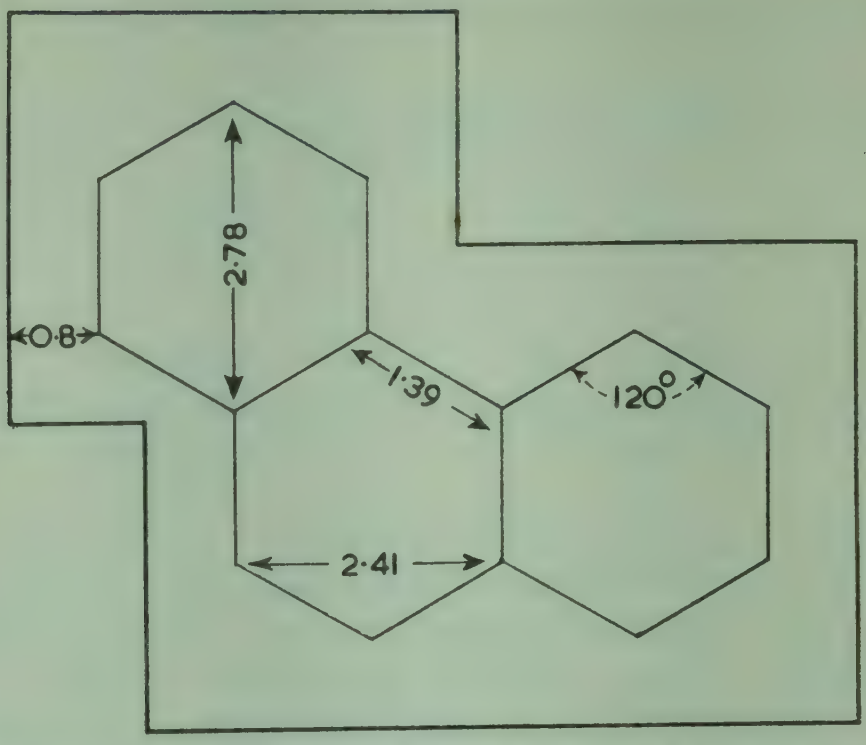



Fig. 8.

It should be pointed out that substances (XIV) to (XVIII) are all completely ionized at pH 7. Analogues of (XVIII) which were not well ionized were not antibacterial. In the larger nuclei with a sufficient area of flatness (such as the acridine nucleus), kationic ionization is the really important limiting factor; but in the smaller nuclei (such as quinoline and pyridine), the importance of the minimal flat area of the molecule is plainly revealed as another limiting factor.

Representations of the kations of 5-aminoacridine (XIV) and its tetrahydro-derivative (XVII) are shown in Plate 2, p. 80. These have been built from Hirschfelder models which accurately reproduce the correct interatomic and van der Waals' distances. The non-coplanarity of the tetrahydro-derivative is plainly seen, especially in the side-view.

Why is the size of the flat area of these kations so nportant? Obviously, increasing the number of atoms in molecule can increase its chances of adsorption. This is ecause the small van der Waals' forces, which unite any om of the molecule to any atom of the bio-receptor rface, become collectively a really strong force when a rge number of atoms are concerned on each side. This rce is opposed by the molecule's kinetic energy of trans- tion which does not increase as the size of the molecule creases.* The bond formed between a kation (on the ug) and an anion (on the bio-receptor) would be a very ansient thing without supplementary bonds of this kind. f all the inorganic kations, the hydrogen ion is the most fficult to desorb, even by other inorganic kations (McCalla 41*a*). Hence it is not surprising that drugs which work an exchange mechanism of this kind should have such itical requirements for adsorption.

It may be asked whether we may expect to obtain werful antibacterials of the acridine type† whenever we sign a molecule having no less than the minimal amount kationic ionization and area of flatness. The answer uld appear to be 'yes', so long as the substance is emically stable. We must except substances like methy- e blue whose reduction potential is so high that it is duced by the metabolic products of some bacteria, such *B. coli*, although it is toxic to a related strain *B. lactis rogenes* which cannot reduce it (the product of reduction not ionized at pH 7). However, there are very definite itations to the number of flat heterocyclic nuclei which yield ionizing amino-derivatives, because every hetero- m in excess of one, drastically reduces basic strength bert, Goldacre and Phillips, 1948). Ways of overcoming

The special requirement of the present series, viz. that the atoms respon- for this adsorption should all lie in one plane, strongly suggests that the ns in the biological surface with which they have to make contact also lie ne plane. Such flat areas are known in the protein and nucleo-protein ecules.

i.e. acting at great dilution against both Gram-positive and Gram-negative ies, the action not being reduced by the presence of protein.

this difficulty have been discussed (Albert, Rubbo and Burvill, 1949).

There is little doubt that the action of *aliphatic* bases which show strong disinfectant properties (i.e. those of $C_{12}$ and over) also depends on the formation of an ionic bond with anionic receptors on the bacteria. Cetyl trimethyl ammonium bromide is a well-known example of this class of compound. In this series, also, the activity increases as the pH is raised, although the drug remains completely ionized throughout (Dunn, 1937; Blubaugh, Botts and Gerwe, 1940; Gershenfeld and Milanick, 1941). Here the resemblance with the heteroaromatic bases (such as the acridines) ends. The aliphatic bases react strongly with serum-protein, being inactivated by their own weight of it, as Rahn showed, whereas the heteroaromatic bases are not influenced by serum at all. The aliphatic bases also differ in the greater speed of their action and in the fact of their producing lysis of the cells.

Such aliphatic sulphonic acids and ethereal sulphates as are antibacterial (i.e. those of $C_{12}$ and over, e.g. sodium tetradecylsulphate) appear to form an ionic bond with an essential kation on the bacterium (cf. Gershenfeld and Milanick, 1941; Miller and Baker, 1940; Putnam and Neurath, 1944).

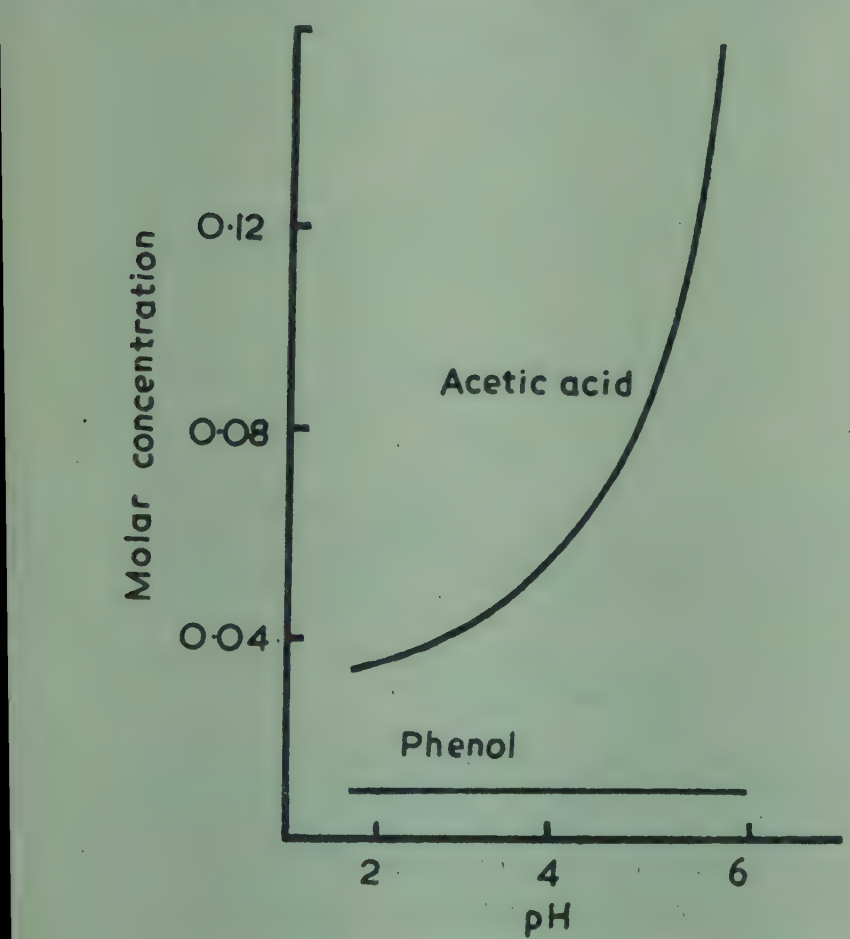
There is considerable evidence of selectivity in this field. For example, *B. coli* is inhibited only by the *ion* of sulphurous acid, but yeast only by the *neutral molecule* (Rahn and Conn, 1944).

### (*d*) *Cases where non-ionization is advantageous but the ion is capable of playing some part*

A number of biological actions involving phenols and other weak acids are known in which the neutral molecule is far more active than the ion, but in which the ion does show some activity.

When working with a weak acid it is usual to find that a constant amount of the substance is required, regardless

f the pH of the medium, provided that the pH is at least units lower than the $pK_a$, thus ensuring that there is no hange in the ionization of the toxic agent. This is well lustrated by Fig. 9, which shows the effect of pH on the oncentrations of phenol and acetic acid required to prevent he growth of various common moulds. Within the pH ange of the experiment (2 to 6) it is seen that a constant mount of phenol is required, but a decreasing amount of cetic acid suffices as the pH drops. This is so because the $pK_a$ of phenol is 9·9, and hence it is non-ionized within he pH range of the experiments, whereas the $pK_a$ of acetic cid is 4·8, and hence it is 90 per cent ionized at pH 5·8 ut only 10 per cent ionized at pH 3·8, and so on.




*Data of Hoffman, Schweitzer and Dalby* (1940 and 1941).

g. 9—The effect of pH on the concentration of phenol ($pK_a$=9·9) and acetic acid ($pK_a$=4·8) required to prevent he growth of common moulds.

This, let us remember, is the typical picture and it has already been outlined in section (*b*) above. However, a rather different picture is revealed in Figs. 10, 11, 12 and 14.

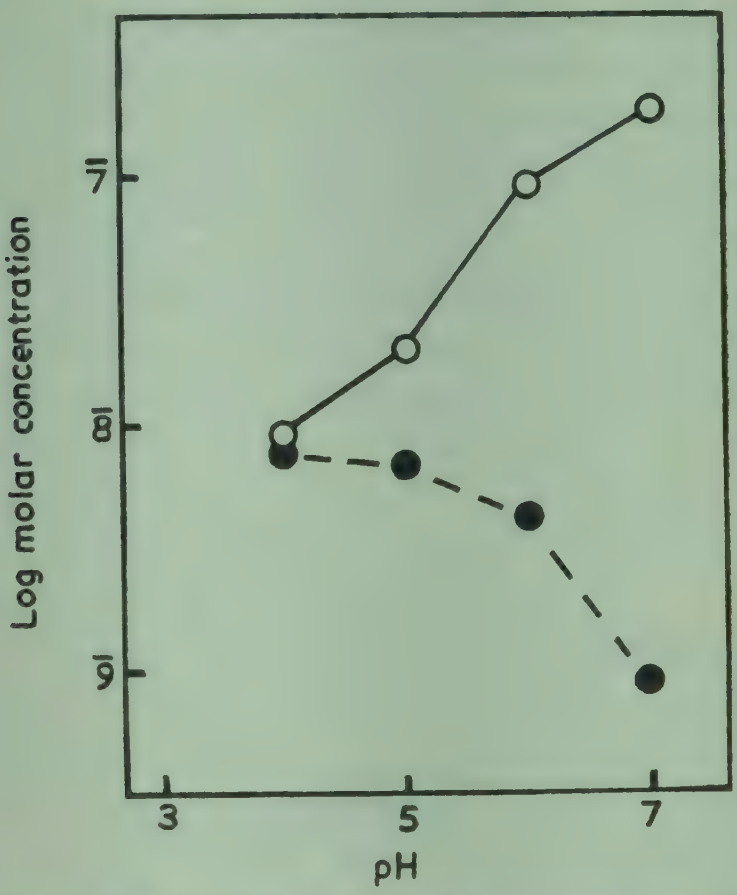
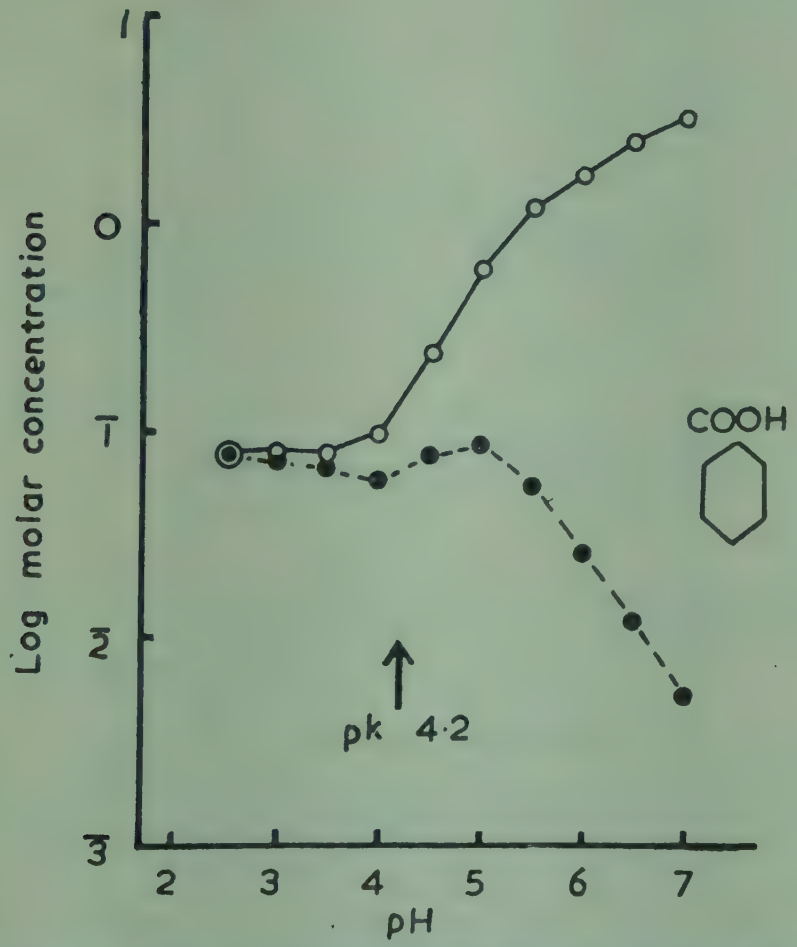



Fig. 10—The effect of pH on the concentration of *p*-aminobenzoic acid required to produce 20 mg. dry weight of *Neurospora crassa* mycelium in 72 hours (*Wyss, Lilly and Leonian*, 1944).

Upper curve: total substance (ions+neutral molecules).
Lower curve: neutral molecules.

Fig. 10 shows the effect of pH on the concentration of *p*-aminobenzoic acid required as a growth-factor for *Neurospora*. The upper curve gives the amount of total substance (ions+neutral molecules) required, the lower curve gives the amount of neutral molecules contained in the total amount. It will be seen that this substance is most economical in use when the substance is at a pH value where it is almost entirely non-ionized. It is not

rprising that, in less acidic solutions, more of the sub-
ance must be taken to get the standard biological
sponse: what is surprising is that this extra amount need
ot be so large as to maintain a standard amount of neutral
olecules. We are forced to conclude that the ions of this
bstance have at least a fraction of the biological activity
the neutral molecules.




g. 11—The effect of pH on the concentrations of benzoic acid required to prevent the growth of *Mucor*.
Upper curve: total substance (ions+neutral molecules).
Lower curve: neutral molecules.
(*Data of Cruess and Richert*, 1929)

The same effect has now been demonstrated for some
ic agents. Fig. 11 shows the effect of pH on the con-
trations of benzoic acid required to prevent the growth

of the common bread-mould, *Mucor*, and Fig. 12 shows the effect of pH on the antifungal action of dinitrophenol upon *Trichoderma*. In both cases the neutral molecules are the most active, but the anions are also active, although in

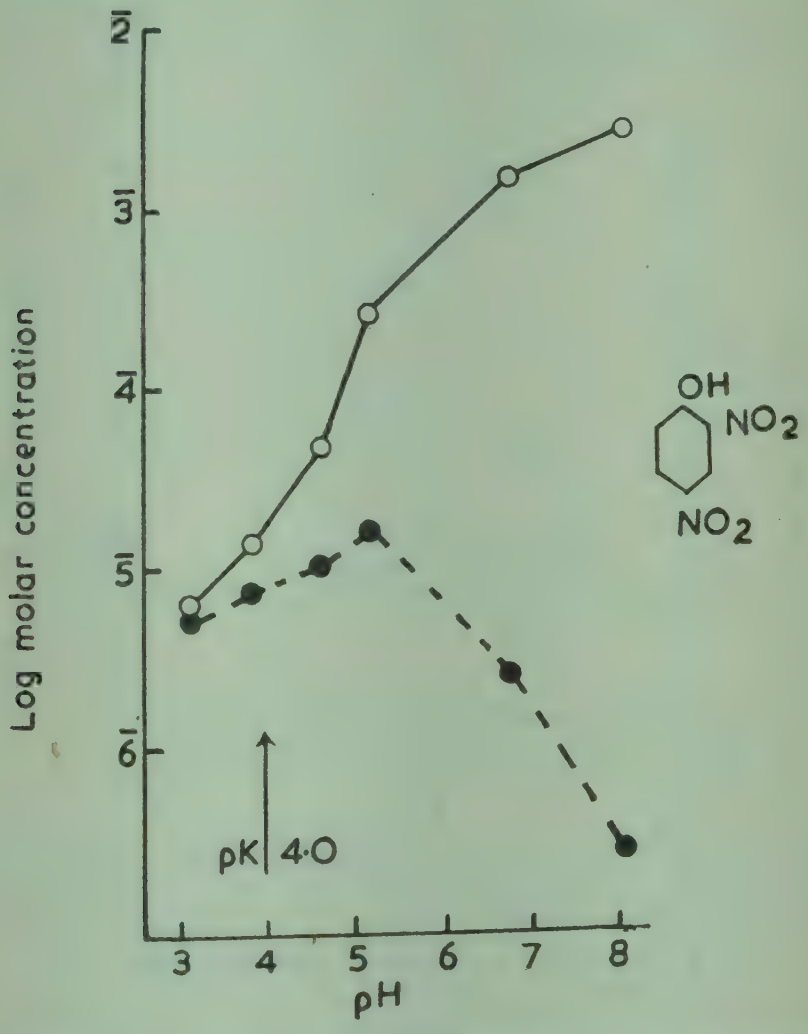
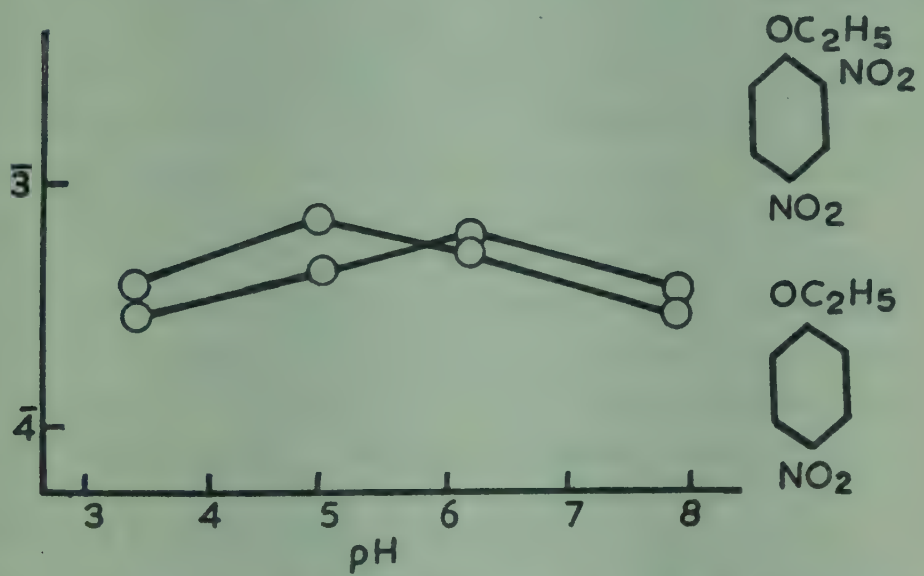



Fig. 12—Effect of pH on the antifungal action of dinitrophenol on *Trichoderma viride* (*Simon and Blackman*, 1948; *Simon*, 1950).
Upper curve: total substance (ions + neutral molecules).
Lower curve: neutral molecules.

a lesser degree. That pH has, of itself, very little effect on *Trichoderma* may be seen from Fig. 13, where the toxic action of two phenolic ethers is shown. These ethers are

osely related to the phenol shown in Fig. 12, but they e, of course, incapable of ionization.*

The anaesthetic action, on the rabbit's cornea, of five cal anaesthetics (cocaine, procaine, stovaine, β-eucaine nd benzylbenzoylecgonine) has been investigated at rious pH values and the intensity of action found to be early proportional to the amount of neutral molecule




g. 13—Comparatively small effect of pH on the antifungal action of some phenolic ethers on *Trichoderma viride* (*Simon and Blackman*, 1949).

esent (Trevan and Boock, 1927). The lethal action of rious organic bases (α-naphthylamine, quinoline, atro-ne, quinine, strychnine, amylamine, coniine and piperi-ne) on paramoecia was shown also to be due principally the neutral molecule (Crane, 1921). However, in both ese examples, it can be calculated that the ion makes a all contribution to the total bio-activity.

A number of examples where the neutral molecule of a bstance is know to be more toxic than the ion have been lected in Table 7, which includes examples of both es (*b*) and (*d*).

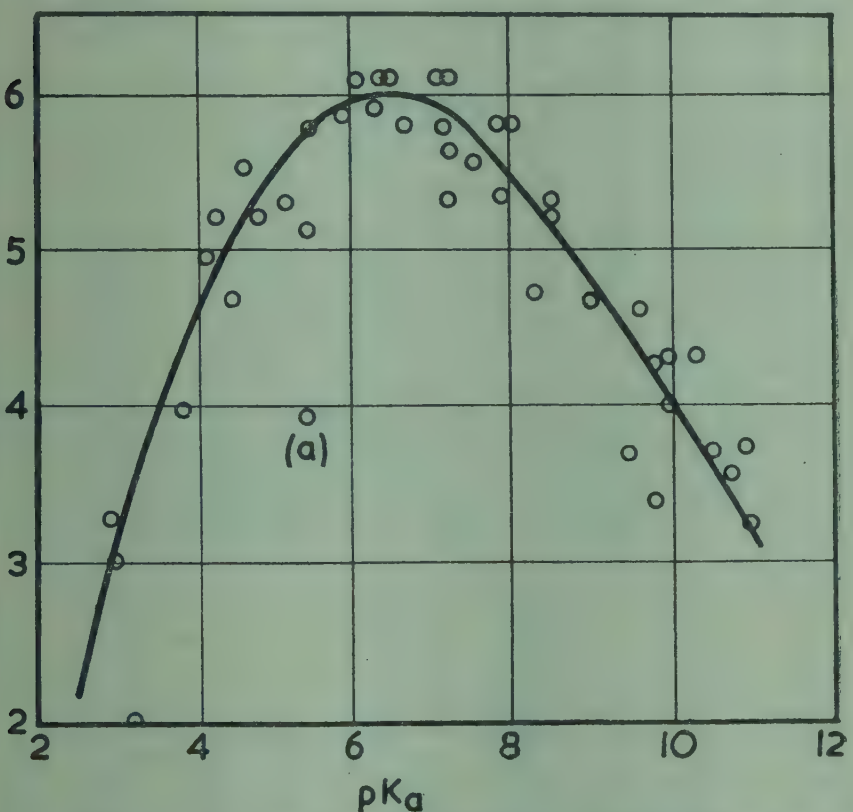
I am grateful to Dr. E. W. Simon of the Department of Agriculture, ord University, for Figs. 2, 9, 10, 11, 12 and 13, and also for his method analysing the results of earlier workers, as used in Figs. 2, 10 and 11.

Another example which we should consider is that of the phenylarsenoxides. We have already seen (Chapter III) that these act by combining with essential —SH groups. *In vitro* experiments show that both ions and neutral molecules have an equal tendency to combine with the —SH groups of cysteine. But when it is a question of rapidly immobilizing trypanosomes and spirochaetes, those phenylarsenoxides which are non-ionized at the pH of the test are enormously more active than those which are ionized (Eagle, 1945). In these experiments, phenylarsenoxides with and without sulphonic- and carboxylic-acid groups were used, and the tests were repeated at several pH values. It is likely that the lethal action of organic arsenicals takes place *inside* the cells, the neutral molecules assisting penetration. Similarly, it was found that arsenious oxide is much more toxic to blow-fly larvae in the form of neutral molecules than as anions (Ricks and Hoskins, 1948).

Fig. 14 introduces us to a series where both anions and neutral molecules appear to be essential for the biological action. This is the sulphonamide series studied by Bell and Roblin (1942). It is well known that sulphonamides which cannot ionize as acids (e.g. sulphaguanidine, cf. also 4:4′-diamino-diphenylsulphone) can be highly antibacterial. However, in a series of 40 sulphonamides studied at pH 7 (Fig. 14), as ionization increases (i.e. as we pass from sulphonamides of $pK_a = 12$ to those of $pK_a = 6$), the antibacterial activity increases considerably. However, further increase in ionization (caused by decrease in $pK_a$) reduces the antibacterial activity. This is a complication not found in the acridine series. Clearly, the most active of the ionizable sulphonamide drugs are those of $pK_a$ around 7, i.e. those that are 50 per cent ionized at the pH of the test.

One suggested explanation (Cowles, 1942) is that the anionic form of the drug is the more potent biologically, but that the action is on the inside of the cell and neutral molecules are essential to secure penetration. Once inside,

e neutral molecules will produce a certain proportion of ions, depending on the $pK_a$ of the drug and the pH of e internal environment* (see equation (1)). If this is true nd it seems unlikely, as 24 hours are allowed for equili- ation), a parabola similar to Fig. 14 should theoretically e produced with any one sulphonamide drug, by varying e pH of the medium in which it is tested. Allowance ould have to be made for variations in the ionization of aminobenzoic acid with pH. This does not appear to ve been tried: *B. coli* would be a suitable organism. The ode of action of sulphonamides will be discussed further Chapter VII.




. 14—Effect on antibacterial action of variation in the $pK_a$ of a series of sulphonamides (*Bell and Roblin*, 1942).

Organism: *B. coli.*

The substances on the left are the most highly ionized (as anions), at the pH of the test (pH 7).

Very little is known of the pH of the inside of bacterial cells, but it is monly believed to lie near to 7 and to change in the same direction as the rnal environment when the latter is changed.

TABLE 7

CASES WHERE THE NEUTRAL MOLECULE OF A WEAK ACID HAS BEEN SHOWN TO BE LESS BIOLOGICALLY ACTIVE THAN THE CORRESPONDING ANION

| *Substance* | *Toxic effect* | *Reference* |
|---|---|---|
| Acetic acid . . . | Inhibition of growth of *saccharomyces*, *salmonella* and *aspergillus* | Levine and Fellers (1940) |
| Normal fatty acids from formic to pelargonic | Inhibition of growth of common moulds | Hoffman, Schweitzer and Dalby (1939) |
| Various fatty acids . . . | Inhibition of growth of *Pseudomonas pyocyanea* | Reid (1932) |
| Acetic, propionic, butyric, chloroacetic and bromopropionic acids | Inhibition of growth of a wine yeast | Huntington and Rahn (1945) |
| Normal saturated fatty acids up to stearic acid | Bactericidal action (the higher fatty acids will only remain dissolved in alkaline media, and hence give the false impression of being more active in alkali) | Eggerth (1929) |
| Acetic and benzoic acids . . | Inhibition of germination of (fungal) spores of *Colletotrichum* | Smith, Walker and Hooker (1946) |
| Acetic and salicylic acids . | Inhibition of growth of *Absidia orchidis* | Dagys and Kaikaryte (1943) |
| Propionic, caproic and caprylic acids | Inhibition of fungal growth (*Aspergillus* and *Trichophyton*) | Wyss, Ludwig and Joiner (1945); Foley, Hermann and Lee (1947) |
| Halogenated fatty acids . . | Inhibition of growth of common moulds | Hoffman, Schweitzer and Dalby (1940) |
| Benzoic acid . . . . | Inhibition of bacterial and fungal growth | Cruess and Richert (1929) |

| | | |
|---|---|---|
| | *charomyces ellipsoideus* | Rahn and Conn (1944) |
| Benzoic, salicylic and *p*-amino-benzoic acids | Inhibition of growth of common moulds | Hoffman, Schweitzer and Dalby (1941, 1942) |
| Benzoic and phenylacetic acids. | Inhibition of bacterial growth (*Staph. aur.* and *B. coli*) | Goshorn and Degering (1938) |
| Barbiturates . . . . | Inhibition of division in fertilized echinoderm eggs | Clowes, Keltch and Krahl (1940) |
| Sulphurous acid . . . | Inhibition of growth of yeast | Rahn and Conn (1944) |
| Hydrofluoric acid . . . | Inhibition of growth of yeast and staphylococci | Roberts and Rahn (1946) |
| Phenylarsenoxides (with and without carboxylic- and sulphonic-acid groups) | Immobilization of spirochaetes and trypanosomes | Eagle (1945) |
| Phenol . . . . | Inhibition of growth of common moulds | Hoffman, Schweitzer and Dalby (1941) |
| Chlorinated phenols (3 examples) | Bactericidal action on *Staph. aureus* | Ordal (1941); Ordal and Deromedi (1943) |
| Chloro- and nitro-phenols . | Inhibition of division of fertilized echinoderm eggs | Krahl and Clowes (1938) |
| 3:5-Dinitro-*o*-cresol . . | Inhibition of growth rate of fungi: *Trichoderma* and *Fusarium* | Brian (1945); Simon (1950) |
| | Mortality of insect eggs (Ephestia and Rhopalosiphum) | Dierick (1943) |
| 2:4-Dinitrophenol . . . | Inhibition of cell division of echinoderm eggs | Tyler and Horowitz (1937) |
| Nitro- and aminonitro-phenols (8 examples) | Inhibition of bacterial growth (*B. coli*) | Cowles and Klotz (1948) |

No matter what element of speculation may exist as to the sulphonamides penetrating as molecules but acting as ions, there is no doubt whatsoever about a series of 16 basic substances of average $pK_a$ 9 examined by Krahl, Keltch and Clowes, 1940. These substances (all of which are local anaesthetics in mammals) were shown to penetrate both eggs and larvae of the sea-urchin *Arbacia* exclusively as neutral molecules, but to act (inside the cell) exclusively as kations.

(*e*) *Conclusion*

The examples cited in this chapter should make it abundantly plain that it is important to know the degree of ionization of every selectively toxic agent which is capable of ionization at a physiological pH value. Having determined this, an attempt should be made to find out in which form the substance is most effective. This should be tried in two ways:

(i) by varying the pH of the medium and plotting toxic action against (*a*) the minimal effective concentration of total substance (as in Fig. 3) and (*b*) the minimal effective concentration of ion (as in Fig. 4.);
(ii) by using modern knowledge of inductive constants to synthesize a few analogues, differing from the original toxic agent in $pK_a$ values only.

The results of (i) and (ii) will be complementary. These experiments take comparatively little time to carry out and can give information that will save months (or years) of work spent in synthesizing compounds on the basis of their merely pictorial similarity to other active substances (to mention only one of the empirical methods in current use).

*CHAPTER FIVE*

# HELATION PHENOMENA AND THE LIVING ELL. FOLIC ACID, 8-HYDROXYQUINOLINE, ND OTHER CARRIERS AND INACTIVATORS OF TRACE METALS

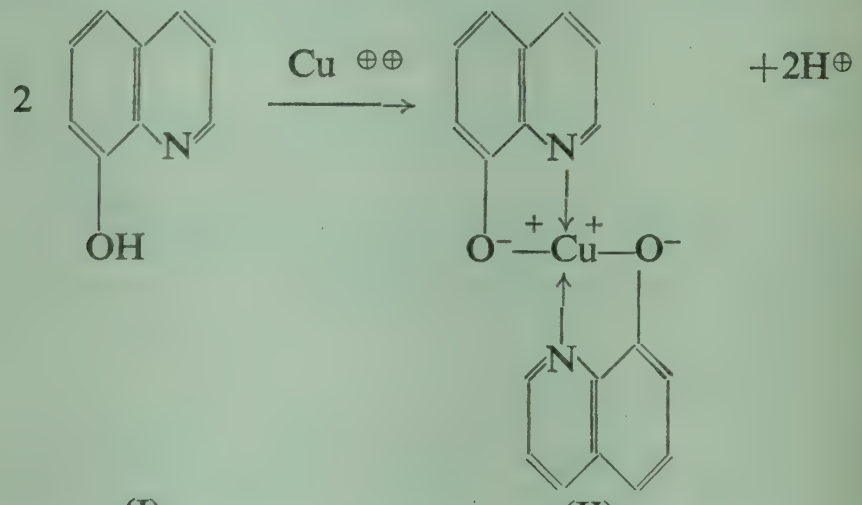
EAVY metals are required in traces by various forms of fe. We have already met some bizarre examples of species equiring vanadium, gallium or molybdenum respectively Chapter I). More widely required metals include zinc, balt, copper, manganese and iron.

) *The nature of chelation*

In previous chapters there has been occasion to discuss e following kinds of bonds: van der Waals', hydrogen, valent and ionic. Now we shall meet a rather different nd of bond, one that is formed between a metallic ion d an organic molecule having two neighbouring groups pable of combining with the metal. Usually one of these oups forms a simple ionic linkage with the metal and the her group co-ordinates with (i.e. inserts a lone pair of ctrons into the outer electronic shell of) the metal. The nding between the metal and the molecule is therefore rtly ionic and partly covalent in character.

The name 'chelation' was coined by Morgan and Drew 920) to describe this phenomenon. The name is derived m *chela* the crab's claw, in allusion to the shape and nction of the metal-seizing pair of groups. A typical mple of chelation is the union of one ion of copper h two molecules of 8-hydroxyquinoline (I) to give one lecule of the 8-hydroxyquinoline-copper complex (II) s two hydrogen ions. The complex is water-repelling, ause every water-attracting group in the system is now sked, viz. the copper ion, the nitrogen atom and the

hydroxyl-group. Such chelated complexes show none of the common reactions of inorganic ions but behave more like organic substances. Complexes, like (II), which are water-repelling, are readily soluble in chloroform and other fat solvents. However, the insertion of extra water-attracting groups produces water-soluble complexes.

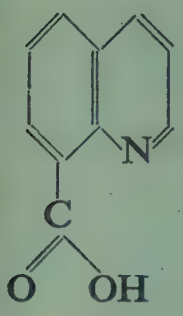



(I) (II)

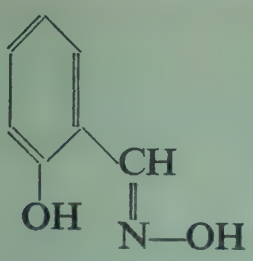
A simple demonstration of chelation is to place 1 ml. of 0·005 *M* copper sulphate in one tube and 1 ml. of 0·005 *M* ferrous sulphate in another tube and to add to each, 10 ml. of 0·01 *M* 8-hydroxyquinoline, which is exactly one equivalent. A yellow precipitate is found in the case of the copper and a greenish-black precipitate in the case of the iron.

There are several other types of structure which bring about chelation. Usually they have an anionic group from which, when non-ionized, the metal ion can displace a hydrogen ion. Near to this group they have a nitrogen, oxygen or sulphur atom with a lone pair of electrons so placed that the metal, in uniting with both groups, can make a strainless ring. Such rings are most stable when they are 6-membered with two double bonds, or 5-membered with one double bond. 8-Hydroxyquinoline (I) gives a 5-membered ring but quinoline-8-carboxylic acid (III) and salicylaldoxime (IV) give 6-membered rings,

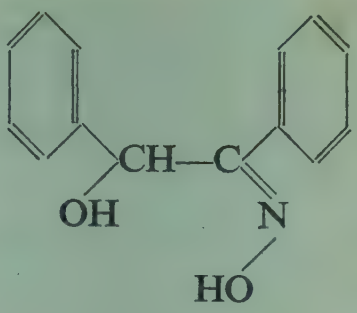
nilar in other respects to (II). Benzoinoxime (V) presents another related type.




(III)

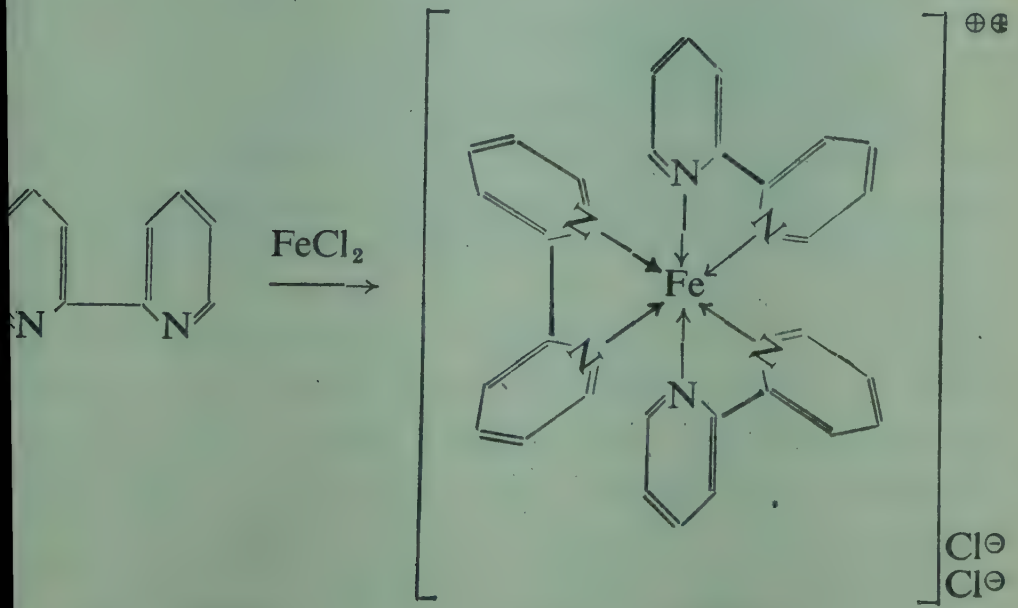



(IV)




(V)

In all the above examples, in fact in the majority of own cases of chelation, each ring is completed by the rmation of one ionic bond and one co-ordinate linkage. ere is, however, another type of chelation in which only -ordinate linkages are involved: no hydrogen ion is split and the metal appears in the final complex accompanied its original chloride (or other gegen) ions. Examples of ents which chelate in this way are 2:2′-dipyridyl (VI) d *o*-phenanthroline; the type of product obtained (VII) red and water-soluble. It should be noted that the iron (VII) is still divalent and has used only auxiliary encies to unite with the reagent.




(VI) (VII)

We should now discuss some quantitative aspects of chelation. Not all chelate complexes are equally stable. Some of these complexes hold their metal so tightly that they do not yield free metallic ions in sufficient quantity to be recognized by the most delicate analytical methods. Others liberate metallic ions plentifully under the same conditions. Often a complex is weak because it has been made with a poor chelating agent; in other cases it is weak because it has been made with a poorly chelatable metal (e.g. Mg). Each complex formed by each chelating agent with each metal has its own stability constant, as defined by the following equation (where $n$ equals the valence of the metal).

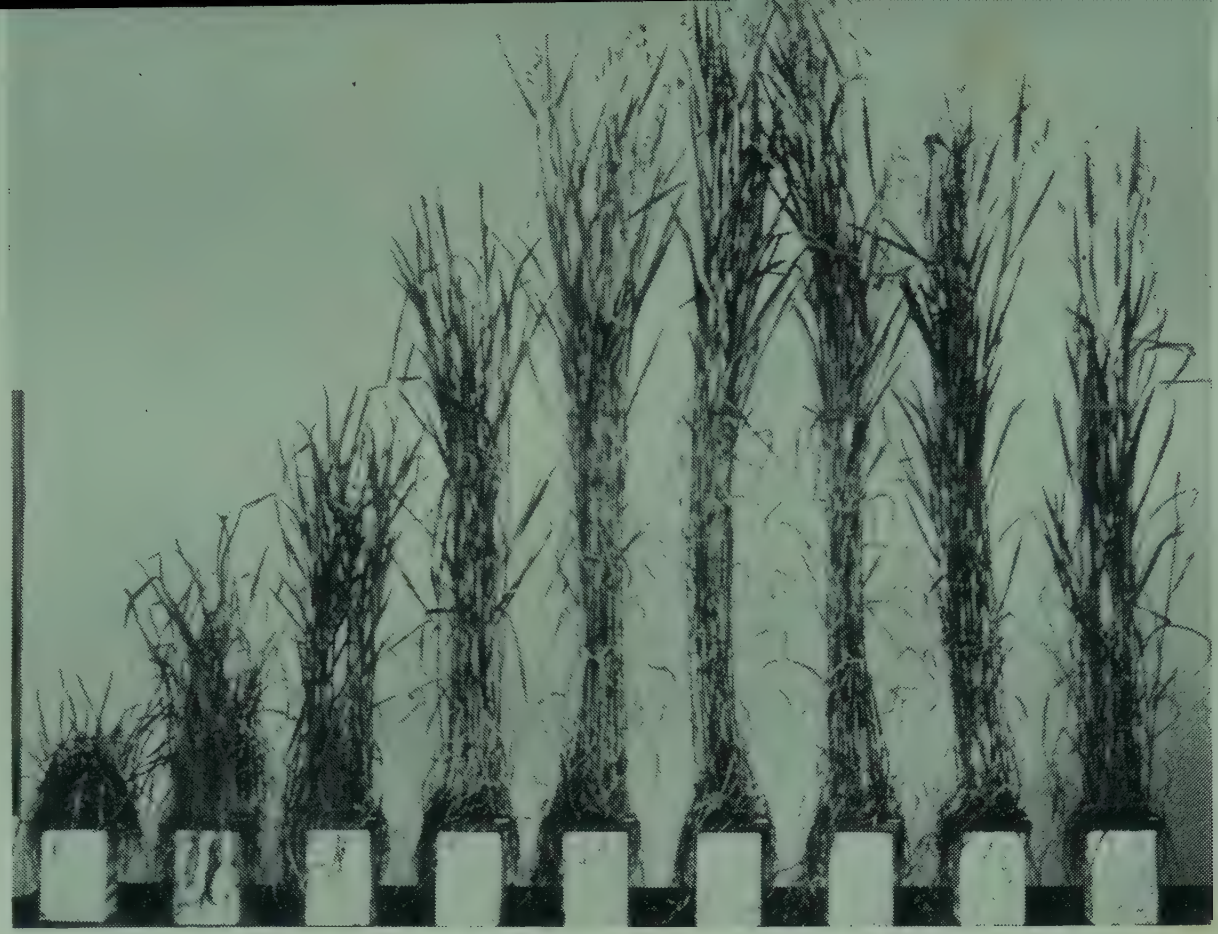
$$K_S = \frac{[\text{chelated metal complex}]}{[\text{metal ions}]\,[\text{chelating agent}]^n}. \qquad \ldots (1)$$

In the case of 8-hydroxyquinoline and copper, this becomes:

$$K_S = \frac{[\text{Cu (8-hydroxyquinoline)}_2]}{[\text{Cu}^{\oplus\oplus}]\,[\text{8-hydroxyquinoline}]^2}. \qquad \ldots (2)$$

There is a formal resemblance to the ionization constants discussed in Chapter IV, but an exponential term has been introduced because of the divalent and trivalent ions with which we are mainly concerned here.

$K_S$ values are too large for convenient use, and are usually recorded as their logarithms. Some examples of log $K_S$ values are: 15 for the equilibrium between glycine and copper (Laitinen *et al.*, 1949); 17 for the ferrous chloride+dipyridyl equilibrium (VI—VII) (Baxendale and George, 1950); 22 for ferrous chloride+*o*-phenanthroline (Lee *et al.*, 1948) and approximately 23 for cupric ion+8-hydroxyquinoline (Rubbo, Albert and Gibson, 1950). These are unusually high figures, and it is hard for the mind to conceive of the minute amount of a metal ion that is in equilibrium with a complex of log $K_S$=16, to say nothing of that quantity, ten million times less, when log $K_S$=23.



3. Effect of copper on the height of oat-seedlings grown in nutrient copper-deficient medium (Piper, 1942). From left to right the quantities of copper present are nil, 3, 6, 10, 20, 100, 500, 2000 and 3000 $\mu$g. per litre

(*see p. 116*)

However, many complexes are less stable, e.g. that between copper and salicylaldehyde-5-sulphonic acid (log $K_S$=9·8) and the cobalt analogue (log $K_S$=5·6) (Calvin and Melchior, 1948).

It has been found that the majority of chelating agents combine with metals in the definite order shown in Table (Mellor and Maley, 1948; Irving and Williams, 1948).* Weak chelating agents can combine only with members at the top of the table. Stronger chelating agents combine more firmly and with more members: most firmly with those at the top of the table, least firmly with those at the bottom.

TABLE 1

STABILITY OF COMPLEXES
THE MELLOR-MALEY SERIES FOR DIVALENT IONS

| | |
|---|---|
| Cu | Most stable |
| Ni | ↓ |
| Co, Zn | |
| Cd | |
| Fe | |
| Mn | |
| Mg | Least stable |

It may well be asked how, if this is the established order, any selective chelation would be possible. One has only to read the books which advertise spot-test reagents to gather that highly selective chelation is possible, but these effects are usually achieved by working at unphysiological pH values or at unbiological temperatures (Albert and Hedhill, 1947). From Fig. 1, for example, it is evident that one could chelate copper at pH 3 without involving cobalt or zinc. At pH 7, all these elements would be chelated, but magnesium would remain in solution. However, these analytical expedients give little help to the selective toxicologist who can seldom control the pH of the species with which he is working. Of more interest to

* Other, simpler, complexing agents such as ammonia and the aliphatic amines behave similarly.

him is the existence of a few chelating agents whose individual properties place them right outside the Mellor-Maley series. Examples of such substances are 2:2′-dipyridyl (VI) and *o*-phenanthroline, which have a quite specific affinity for iron. These substances will chelate iron without affecting ions higher up in the series. It would be of great value, in selective toxicity, if more substances of this kind were sought, substances which would be truly specific for given ions at pH 7. Copper is so essential for both animal and plant life that the use of existing chelating agents is bound to be limited.

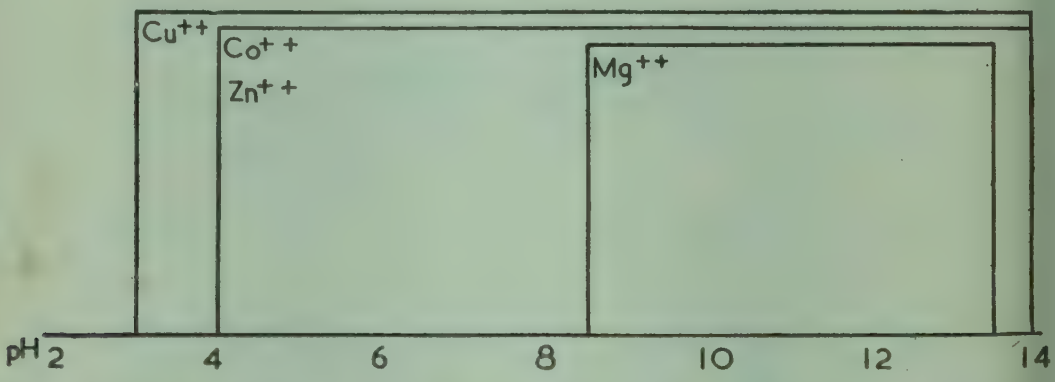
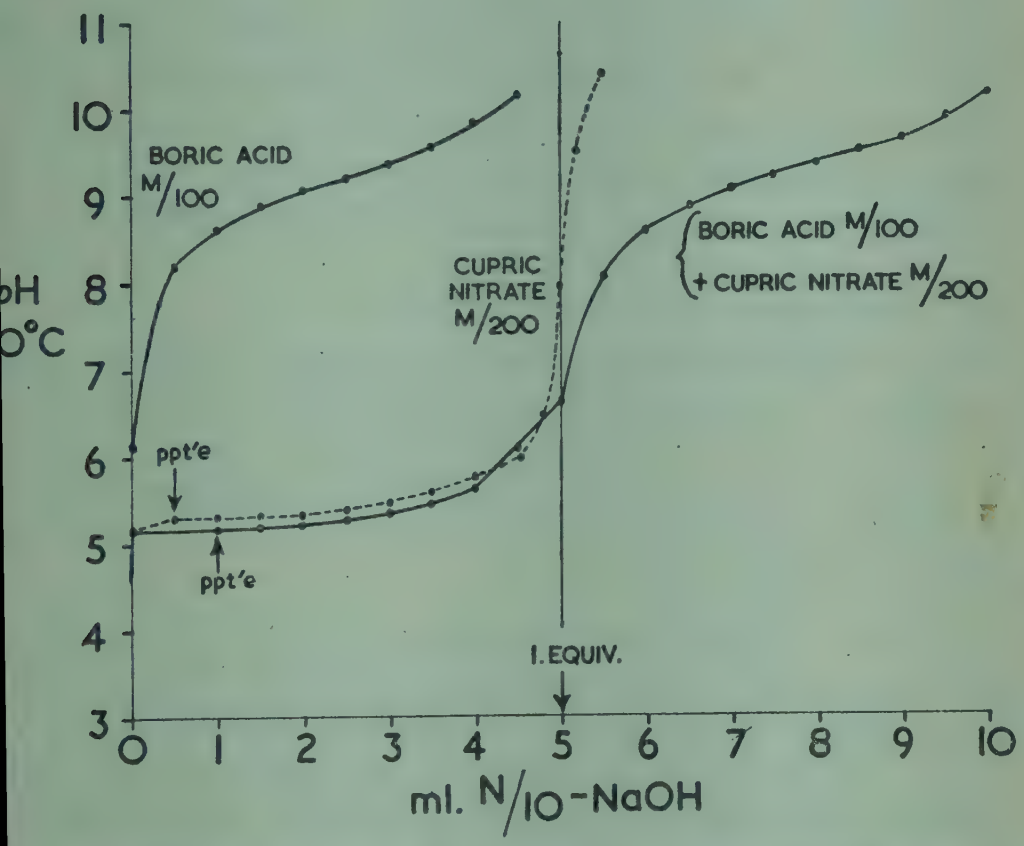



Fig. 1—Range of pH between which 8-hydroxyquinoline (0·01 M-) can precipitate various metallic ions from approx. 0·003 M-solutions. *Fleck and Ward* (1933)

Let us consider the case where a chelating agent can combine with the ions of two metals but is present in insufficient quantity to combine with both. These are competitive conditions. Provided the metallic ions are widely enough separated in the Mellor-Maley series, the agent will combine with that ion which is highest up in the series. This can easily be shown experimentally. If 1 ml. each of 0·005 *M* copper sulphate and ferrous sulphate are placed in a test-tube and 10 ml. of 0·01 *M* oxine added, there is only enough oxine to combine with one of the metals. Hence it can be seen from the colour whether it combines with the iron (greenish-black), copper (yellow) or a mixture of both (green). As copper is so much higher

p in the series than iron, we should expect it to combine xclusively with the copper; and that is exactly what appens. That there is plenty of ferrous iron present, can e seen from the brilliant red colour that is produced when ne equivalent of *o*-phenanthroline is added finally to the me tube.




g. 2—Example of the application of potentiometry in the investigation of chelation. No chelation is found in the present case (Boric Acid and Copper ions). Contrast with Fig. 3.

The detection of chelation and the measurement of the propriate $K_S$ values present many problems. When the elated complex is insoluble, the appearance of a chloro-m-soluble precipitate on mixing the two components is nfirmatory. Sometimes, too, there is a colour change. nen there is neither precipitate nor colour change, ourse can be had to potentiometric titration. Examples this procedure are given in Figs. 2 and 3. In brief

the acidic group of the suspected chelating agent is titrated with alkali, noting the pH obtained as each tenth of an equivalent is added. Then an equivalent amount of copper nitrate solution is titrated. Finally, one titrates a mixture of the suspected chelating agent and copper nitrate. If there is no complex formed, the new curve follows, in turn, the two original curves (see Fig. 2, where boric acid and copper nitrate have been titrated in this way). This family of curves should be compared with Fig. 3 in which chelation is plainly demonstrated when 4-hydroxypteridine and cobalt nitrate are mixed. The changed pH of precipitation should particularly be noted.

(*b*) *Trace-metals and chelation in nature*

Zinc deficiency causes serious disease in grapes, apples and citrus fruits and is responsible for poor yields of cereals in certain areas. It is a constituent of the enzyme carbonic anhydrase, which hastens the equilibrium between carbonic acid and carbon dioxide, and is essential to all the higher forms of animal life.

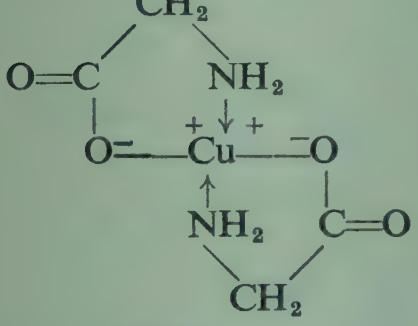
Cobalt is an essential constituent of the anti-pernicious anaemia factor, also known as vitamin $B_{12}$, which is produced by many species of bacteria and is required by man. Cobalt deficiency occurs in pastures when the cobalt-content falls below 0·2 parts per million: sheep reared on such pastures become anaemic and weak.

Copper is an essential constituent of several enzymes such as polyphenoloxidase (tyrosinase) which is found in plants, worms and arthropods. Copper takes the place of iron in the respiratory pigment of certain molluscs. Until recognized as such, copper deficiency was the cause of many a crop failure in reclaimed areas of Holland and Denmark. Copper and manganese are known to be essential for the development of the chlorophyll molecule. Copper is also known (and manganese suspected) to be essential for the production of haemoglobin in man. Plate 3 shows the effect of traces of copper on oat seedlings grown in a

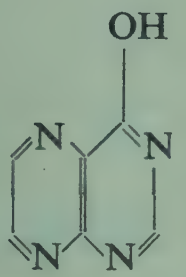
pper-deficient medium. It is evident that too little pper is as bad for growth as too much.

Iron is a constituent of the various porphyrin enzymes ytochromes, peroxidase, catalase) as well as of haemo-obin. Two other important iron compounds occur in ie human body, ferritin which transfers iron from the owel to the tissues and transferrin* which reduces the ncentration of ferrous ions in the blood to vanishing-oint in order to prevent iron-poisoning.

Of all forms of life, probably none has been so little xplored for its requirements of trace elements as the acteria pathogenic to man. A survey of the literature iscloses only that iron is considered essential for a number f pathogenic species. Apart from questions of essen-ality, however, it is known that zinc is stimulating for the iphtheria organism, iron and manganese for the anthrax acillus and lead for the gonococcus.



(VIII)



(IX)

Two classes of chelating agents utilizing trace-metals nature have long been recognized, the porphyrins and mino-acids. An example of the latter class is furnished the glycine-copper complex (VIII). Quite recently, have found that riboflavine, the purines and pteridines e capable of chelation. Hence the possibility arises that ese are new classes of compounds responsible for trans-rting the heavy metals in normal metabolism. In Fig. 3

* Originally isolated from eggs, and called conalbumin, this $\beta_1$ globulin can lower the concentration of ferrous ions in a medium that bacteria will not w in it (Schade and Caroline, 1944). It is also known as siderophyllin.

the potentiometric titration of 4-hydroxypteridine (IX) is shown. It is quite evident that this substance chelates strongly with cobalt up to pH 8·6. A comparison of the formula of this substance with 8-hydroxyquinoline (I) is instructive. As would be expected, the complexes formed by (IX) and by folic acid are water-soluble.

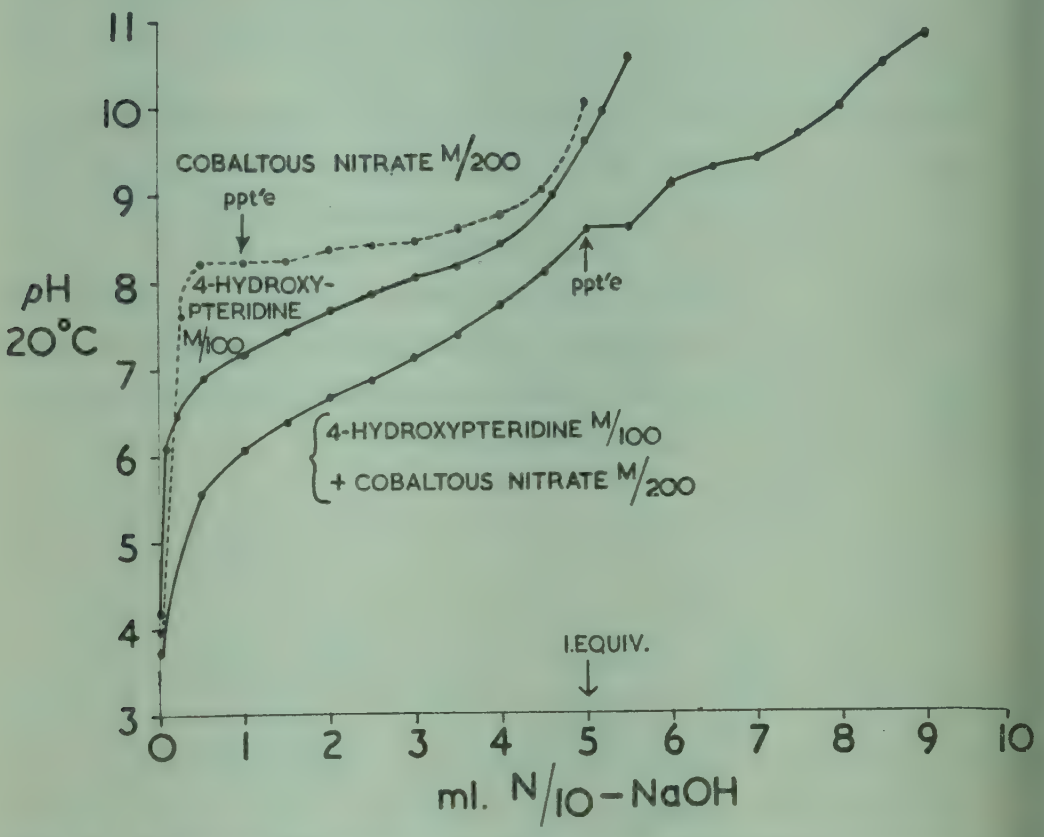



Fig. 3—4-Hydroxypteridine. Example of the detection of chelation by potentiometry. Complex formation exists up to pH 8·6 at least.

Facts such as these make it plain that in nature a constant competition is going on between chelating substances and the ions of metals. In tissues where powerful chelating groups exist, a liberal supply of ions is necessary if any useful proportion of them is to remain free. Where nature's chelating groups are weaker, they must be provided abundantly if there is a surplus of ions to be mopped up. Various chelating agents will be competing with one another for

tain ions, particularly those near the top of the Mellor-aley series (Table 1). Dynamic equilibria of this kind e essential for normal metabolism and it is doubtful if e can continue when they are seriously disturbed.

Many diseases of plants and animals are traceable to ıladjustments in the balance between metals. Soybean ants, treated with an excess of manganese, quickly develop ns of iron deficiency and this can be corrected by dosing em with iron. If, on the contrary, they are grown in soil at is too rich in iron, they develop manganese deficiency omers and Shive, 1942). In Britain, various pastures use the serious economic disease in lambs called *swayck*. This is relieved by copper, and is associated with the esence of excessive amounts of zinc or lead in the grass. her pastures, called *teart*, are rich in molybdenum and use the signs of copper deficiency in sheep, relieved by e feeding of copper (Ferguson, Lewis and Watson, 1943). cess of zinc in the diet of rats causes an anaemia relieved copper (Smith and Larson, 1946). Land in Holland ich had been dressed with copper to prevent 'reclamaı disease' (copper deficiency) has often produced crops th a marked deficiency of manganese, although the soil d the normal content of manganese.

These antagonisms are reminiscent of the simple antanism (calcium versus potassium) which is responsible regulating the beat of the heart, as Ringer showed in 80. A similar antagonism between magnesium and cium is concerned in the contractility of muscle. rtain bacteria (*Lactobacillus casei* and *Streptococcus calis*) have been shown to be injured by traces of dium or ammonium ions, but only when grown on a tassium-deficient medium. These bacteria, however, are e to utilize rubidium in place of potassium (MacLeod d Snell, 1948).

In many of these antagonisms we can see the inorganic uivalent of the metabolite analogues discussed in apter III.

So potent are the effects of various trace elements, that the ability of media to support life is due to quantities so small that their presence may not be suspected. Even a substance that is 99·99 per cent pure has 600,000,000,000,000,000 foreign molecules in each gram.*

Bacteriological media are frequently rich in traces of heavy metals. One brand of peptone† advertises an iron content of 100 parts per million and a copper content of 30 p.p.m. Another popular brand was found to contain between 10 and 100 p.p.m. of aluminium, copper, iron, manganese, molybdenum, nickel, silver, tin and zinc. A brand of meat extract, much used by bacteriologists, contained similar quantities of aluminium, copper, iron, manganese, molybdenum, tin and zinc (Oertel, private communication). 'Analar' sodium chloride contains spectrographically detectable amounts of silver and copper.

Cobalt is not so widespread, but it is by no means absent. A synthetic medium composed of glucose, ammonia, nitrate and phosphate assayed 0·33 p.p.m. of cobalt; a bacteriological peptone contained 0·25 p.p.m. and meat extract 0·08 p.p.m. (Lee and Dewey, private communication).

Chelation has been used to obtain media deficient in several metallic ions; to this medium, corrective additions may be made, leaving it deficient in the ion whose effect is to be studied. This field was pioneered by Waring and Werkman (1942), who extracted media alternately with 8-hydroxyquinoline and with chloroform in order to study iron deficiency in bacteria; and by Piper (1942), who obtained his copper-deficient media for the oat plants shown in Plate 3, by using dithizone similarly. 2:2·-Dipyridyl has been added to media in which *Clostridium acetobutylicum* is growing in order to lower the content of ferrous ions and thus increase riboflavine production

* Calculated from the Avogadro number ($6 \times 10^{23}$ particles per mole of any substance) and an assumed average molecular weight of 100.

† Oxoid bacteriological peptone.

Hickey, 1945); a similar process is used for the commercial roduction of riboflavine.

*) Selective toxicity through chelation*

Reference to Plate 3 will suggest two ways in which the ell's need for trace-metals could be utilized in designing oxic agents. With reference to the right-hand portion of he curve traced by the living plants, one might design electively toxic agents by using metallic salts in concen-rations which are injurious to the uneconomic species ut harmless to the economic species. A great deal of netallotherapy of this kind has been attempted in the past, g. the spraying of orchards and vineyards with copper reparations, and the injecting of gold salts into patients uffering from rheumatoid arthritis. An interesting variant vas the attempt to catalyse the destruction of bacterial oxins in the blood-stream by traces of copper or zinc Petherick and Singer, 1944). It is not very likely that traightforward metallotherapy has much to offer us; it as been intensely explored for so many years and the esults, on the whole, have been disappointing.

The left-hand portion of the curve in Plate 3 suggests he use of chelating agents as selectively toxic substances emoving essential trace-metals from uneconomic species nd leaving the economic species with its full requirement. his could be accomplished by introducing into the mole-ule such properties as would favour the agent being istributed principally to the uneconomic species (cf. the ase of sulphuric acid in Chapter I and of phenylpanto-henone in Chapter III). Alternatively, a chelating agent ould be sought which would chelate a metal that was vital nly to the uneconomic species. The latter method would e difficult in the light of our present knowledge, but not y any means impossible. The use of BAL (dimercaprol) a complex-former in metallic poisoning gives us reason believe that even chelating agents with wide affini-es could be used in the chemotherapy of blood-borne

infections. Injections of BAL have cured patients who were poisoned by gold, mercury or arsenic* (Russell, Green and Wand, 1948; Peters *et al.*, 1947). Only transient toxic symptoms were observed, although BAL can inactivate many metal-containing enzymes (Webb and van Heyningen, 1947; Barron, Miller and Meyer, 1947).

The use of dithiocarbamic acid and of dithiuram sulphides as selectively toxic agents in agriculture represents a successful (although unconscious) application of chelating agents. 8-Hydroxyquinoline, however, provides the most thoroughly investigated example.

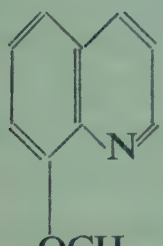
8-Hydroxyquinoline (I) has been used clinically for fifty years as a powerful antibacterial in wounds. Its mode of action remained unknown until quite recently. It is true that Hata (1932) suggested that 8-hydroxyquinoline owes its antibacterial properties to the combination, in one molecule, of phenol and quinoline. But this notion cannot be seriously entertained because neither phenol nor quinoline are active at a dilution of 1 in 500, whereas 8-hydroxyquinoline is active at a dilution of 1 in 700,000. Moreover, it is unreasonable to expect electronic distributions appropriate for evoking a given physiological effect in one molecule, to be compatible with those appropriate in another type of molecule, let alone to be mutually enhancing!†

Because 8-hydroxyquinoline acts on bacteria in very high dilution, it is likely to be interfering with a particular metabolic requirement of these organisms. That this specific action might well take the form of chelation between the drug and essential trace-metals, was put forward by the author on the grounds that 8-hydroxyquinoline can combine powerfully at pH 7·3 with the following biologically important ions: $Cu^{++}$, $Zn^{++}$, $Co^{++}$, $Fe^{++}$, $Fe^{+++}$, $Mn^{++}$ and $MoO_2^{++}$ (Albert, 1944; Albert and Magrath, 1947). About the same time, an American

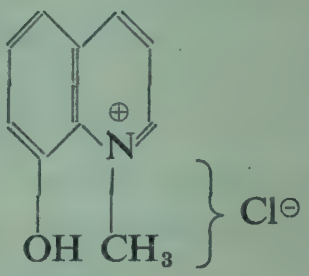
* Also, in animals, nickel, antimony, bismuth and chromium.
† This error of logic is still quite commonly indulged in!

orker suggested that 8-hydroxyquinoline acts against ngi by chelation (Zentmyer, 1943). The antibacterial tion was investigated further by a team in Australia lbert, Rubbo, Goldacre and Balfour, 1947).

Firstly, it was found that of the seven possible mono-ydroxyquinolines, six of them did not chelate, and these ere not antibacterial. On the other hand, 8-hydroxy-uinoline chelated powerfully and was powerfully anti-acterial. Secondly, it was found that blocking either the xygen or the nitrogen of 8-hydroxyquinoline (as in X nd XI respectively) prevented chelation and also abolished ntibacterial activity.

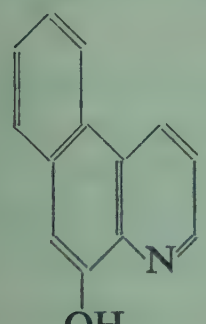



(X)




(XI)

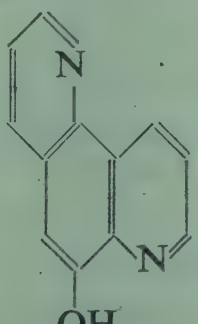
Thirdly, the characteristic groups of 8-hydroxyquinoline iz. a hydroxyl-group *peri* to a ring-nitrogen) produced a rong chelating and strong antibacterial effect in other uclei also. This is shown in formulae (XII), (XIII) and XIV). The dilution just preventing growth of streptococci,




(XII)

-Hydroxy-5 : 6-benz-quinoline

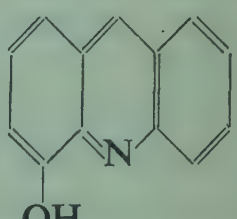
(M/200,000)




(XIII)

6-Hydroxy-*m*-phenanthroline

(M/100,000)




(XIV)

1-Hydroxyacridine

(M/8,000)

after 48 hours at 37° (medium: broth at pH 7·3), is given in brackets (8-hydroxyquinoline, for comparison, is M/100,000).

It can be seen that 1-hydroxyacridine (XIV) is a weaker antibacterial than the others and further investigation showed that a substituent in the 2-position (even a methyl-group) of 8-hydroxyquinoline considerably lowered the antibacterial action, although such groups could be placed in other positions without reducing the activity. Clearly, there is some steric effect exerted by this position, suggesting that 8-hydroxyquinoline either acts at a surface or has to penetrate a membrane before it can act. In spite of its comparatively low activity, 1-hydroxyacridine is considerably more antibacterial than other acridines of comparably poor ionization (see Appendix I to this book).*

Among a total of fifty derivatives and analogues of 8-hydroxyquinoline, the only other substances in which antibacterial action did not run parallel to the degree of chelation were those having a fully dissociated anionic group. Such substances (e.g. 8-hydroxyquinoline-5-sulphonic and -5-carboxylic acids) were strong chelating agents but proved to be not at all antibacterial. As was explained in Chapter IV, an anionic group can prevent a molecule from penetrating the cell-membrane. This supports the location of the site of action of 8-hydroxyquinoline suggested above, viz. in the bacterium and not in the bulk of the medium.

Because 8-hydroxyquinoline is obviously acting by chelation, a reasonable hypothesis would be that it is removing a trace-metal essential for metabolism. Such a hypothesis implies that the inhibited bacterial metabolism could be restored if the missing metal were added. Hence it should be possible to find out the nature of this essential metal by adding various metallic ions and seeing which one specifically reverses the bacteriostasis. Unfortunately,

* As a matter of fact, it was the anomalously high activity of this substance which first suggested to us the mode of action of 8-hydroxyquinoline.

th Gram-positive organisms, the class against which hydroxyquinoline is most active, death occurs so quickly at reversal experiments can only be conducted if the etal is added immediately after the drug. When such experiment was carried out, it was found that the only n which could reverse the action of 8-hydroxyquinoline as that of cobalt. Hence a reasonable deduction would that cobalt is an essential trace-metal for the many ram-positive organisms against which 8-hydroxyquino-ne is so active and that this drug acts by making the balt unavailable to the bacteria.

A proper objection to this conclusion is that cobalt may activate the 8-hydroxyquinoline by chelating with it fore it can be taken up by the bacterium. Yet, if this were , why is nickel not a reversing agent, as it is non-toxic d oxine has a greater affinity for nickel than for cobalt.*

Since this work was done, it has become known that balt must be essential for the following micro-organisms ll of which produce vitamin $B_{12}$, which contains cobalt): *acillus subtilis*, *Lactobacillus arabinosus*, *Streptomyces iseus* and *Mycobacterium smegmatis*. As was shown ove, traces of cobalt are likely to be found in all media.

The importance of this presumed interaction between ine and cobalt in bacteria is supplemented by more cent work which has shifted the emphasis from a *depletion* an *excess* of metallic ions as the more important cause of e toxicity of oxine (Rubbo, Albert and Burvill, 1950). It w appears that, regardless of whether or not oxine is with-awing a somewhat essential trace-metal from the organ-ms, its rapid lethal action is caused by a form of metallic isoning, apparently iron poisoning. When *Staphylococcus reus* is placed in double glass-distilled water, it does not w, but nevertheless remains viable for a day, as can be monstrated by plating-out. The addition of some hydroxyquinoline (final concentration, M/200,000 or

* That 8-hydroxyquinoline forms a tighter complex with copper (and with kel) than with cobalt has been demonstrated by determination of the ility constants (Rubbo ,Albert and Gibson, 1950).

M/200) does not alter this state of affairs unless an iron salt (M/50,000) is added at the same time, whereupon the organism instantly dies. In place of water, nutrient media can be used in this experiment, provided that they are made poor in metals by extraction with oxine and chloroform. The iron salts are non-toxic in the absence of 8-hydroxyquinoline.

What is the interpretation of this experiment? It could be that the drug removes a 'guardian metal' (say, cobalt) that is protecting an essential thiol group from autoxidation, no harm arising unless a toxic metal (e.g. iron) which is capable of promoting autoxidation is added. This suggestion brings to mind the experiment of Baur and Preis (1936) in which cysteine is protected from copper-catalysed autoxidation when cobalt is added.

Iron poisoning (hyperferraemia) is a well-known phenomenon in children who accidentally swallow tablets of ferrous sulphate (Forbes, 1947; Thomson, 1947), but it has only recently been discussed in a microbiological connexion. Racker and Krimsky (1947) have suggested that encephalomyelitis virus, which becomes richer in iron the more it is purified, causes the typical brain injury by transporting ferrous ions across the blood-brain barrier which is normally impervious to them. These authors brought their hypothesis to the biochemical level by showing that ferrous ions strongly and specifically inhibit the oxidation of phosphoglyceraldehyde in normal glycolysis (Racker and Krimsky, 1948).

Another interesting type of experiment which has been repeatedly carried out with *Staph. aureus* and *Strept. pyogenes* is exemplified in Table 2. This table shows that, whereas dilute solutions of 8-hydroxyquinoline are rapidly lethal, stronger solutions are quite compatible with vigorous growth of the organism.* It may well be that it is the

* Such inversions of the usual relation between concentration and toxic effect are exceedingly rare but have been demonstrated in another chelating agent, tetramethylthiuram monosulphide, acting on the fungal spores of *Venturia* (Montgomery and Shaw, 1943).

## TABLE 2

BACTERICIDAL ACTION OF 8-HYDROXYQUINOLINE

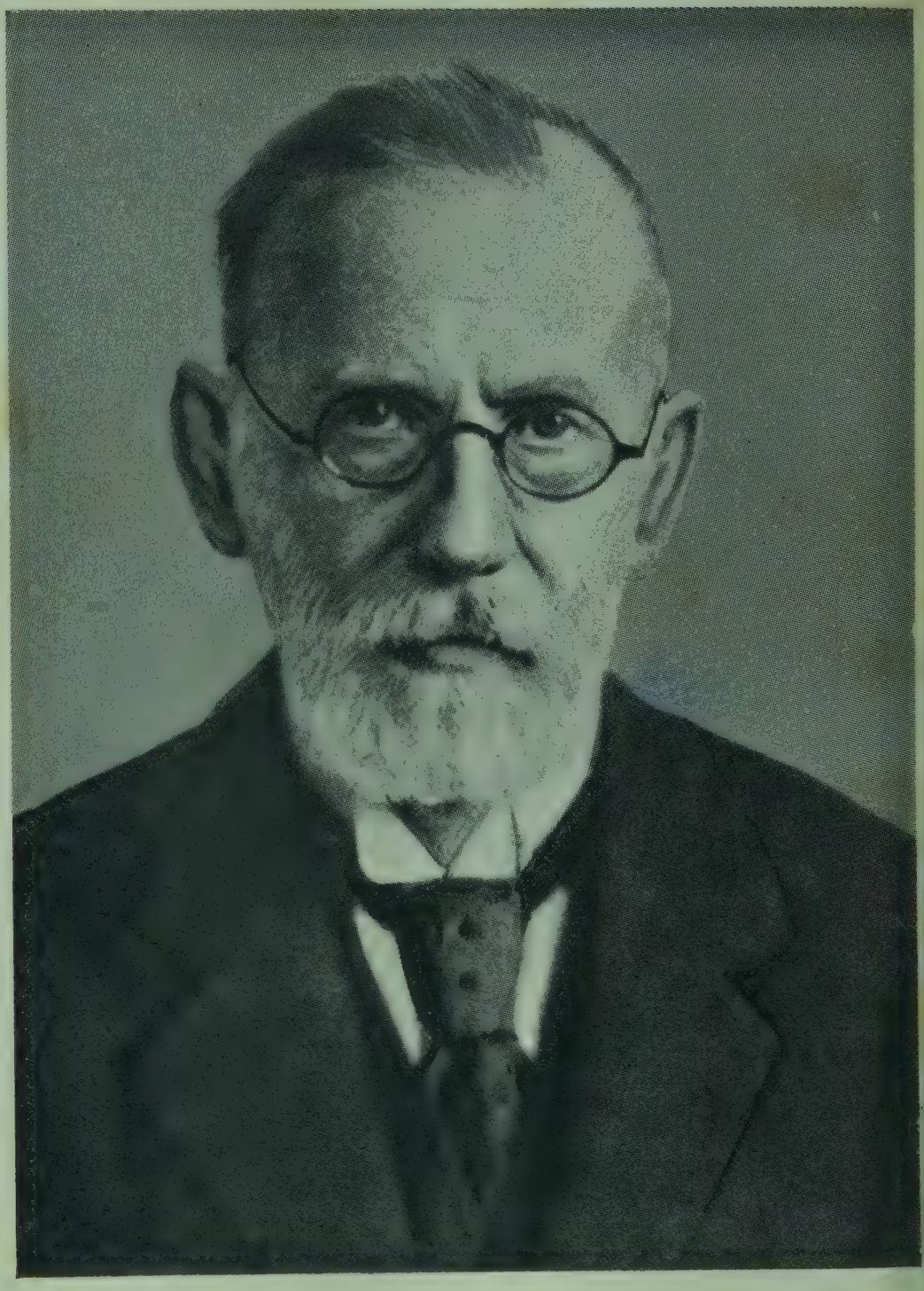
Least bactericidal concentration for *Strept. pyogenes* at 37° (medium: broth, pH 7·3)

| *Time of exposure to drug* | *No drug* | M/102,000 | M/51,000 | M/26,000 | M/13,000 | M/6,400 |
|---|---|---|---|---|---|---|
| 0 hour . . . | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| 3 hours . . . | ++++ | − | − | − | ++ | +++ |
| 20 hours . . . | ++++ | − | − | − | + | +++ |

(Albert, Gibson and Rubbo, *in the press*.)

1 : 1 iron-hydroxyquinoline complex or free ferrous ions (formed from it by hydrolysis inside the cell) which kill the bacteria. However, when an excess of oxine is present only the 1 : 2 complex can be formed, according to the mass action law (see equation (1)), and this is very likely to be non-toxic.

These data are complex and do not yet tell us all we wish to know. However, they serve to illustrate the potentialities that exist for applying chelation to selectively toxic ends.



4. Paul Ehrlich
1854—1915

*CHAPTER SIX*

# HE HISTORY OF CHEMOTHERAPY FROM THE TANDPOINT OF THE UNDERLYING SCIENTIFIC PRINCIPLES

N the previous chapters we have reviewed the mode of tion of selectively toxic agents from the standpoint of esent-day knowledge. It should now be interesting to ke a particular branch of this subject and to trace the scovery of its governing principles. Chemotherapy is the ost suitable example for this purpose because its govern- g principles are somewhat better established than those any other branch of selective toxicity.

This chapter will be concerned mainly with matters of ıre science. The utilitarian angle, that is to say, the mance of the discovery and use of drugs which are now ıring diseases previously considered hopeless, need not gone into here: it will be only too well known to the ader. A list of the historically most important chemo- erapeutic agents is given in Table 1. If reference is ade to this table, the complete picture can be recon- ructed from two interlocking strands: the discovery of ilitarian drugs and the discovery of the principles which d to them or explained their mode of action.

The word 'chemotherapy' was coined by Ehrlich to scribe the cure of an infectious disease without injury the host. Hence, it seems natural that a historical count, such as this, should begin with Ehrlich (Plate 4). ctually, a few chemotherapeutic agents were known fore his time. These were cinchona and ipecacuanha, r the cure of malaria and amoebic dysentery respectively, d mercury for the alleviation of the symptoms of syphilis. ercury began to be used in this way in the sixteenth ntury, cinchona and ipecacuanha in the seventeenth.

## TABLE 1

THE DISCOVERY OF CHEMOTHERAPEUTIC SUBSTANCES

| *Date* | *Drug* | *Condition in which used* | *By whom introduced* |
|---|---|---|---|
| — | Cinchona | Malaria | Brought to Europe from South America in 17th century |
| — | Ipecacuanha | Amoebiasis | |
| 1904 | Trypan red | Trypanosomiasis (mouse)* | Ehrlich and Shiga (synthesized by Weinberg) |
| 1905 | 'Atoxyl' | Trypanosomiasis | Thomas and Breinl (synthesized by Béchamp, 1860; structure elucidated by Ehrlich and Bertheim, 1907) |
| 1907 | Antimony (tartar emetic) | Trypanosomiasis* | Plimmer and Thompson (mouse); Manson (man) |
| 1910 | 'Salvarsan' | Syphilis | Ehrlich and Hata (synthesis reported by Ehrlich and Bertheim, 1912; D.R.P. 224,953 (1909) to Hoechst) |
| 1911 | 'Optochin' | Pneumonia (mouse)* | Morgenroth and Levy |
| 1912 | Antimony (tartar emetic) | Leishmaniasis | Vianna |
| 1912 | 'Trypaflavin' (acriflavine) | Trypanosomiasis (mouse)* | Ehrlich (synthesized by Benda) |
| 1912 | Emetine | Amoebiasis† | Rogers |
| 1913 | Acriflavine | Sepsis of wounds | Browning |
| 1918 | Antimony (tartar emetic) | Schistosomiasis† | Christopherson |

| | | | |
|---|---|---|---|
| | (suramin) | | |
| 1921 | Bismuth . . | Syphilis† . . . . . | Sazerac and Levaditi |
| 1926 | 'Plasmoquine' . | Malaria . . . . . | Roehl (synthesized by Schulemann, Schönhöfer and Wingler, as reported by them in 1932) |
| 1930 | 'Carbarsone' . | Amoebiasis . . . . | Leake, Koch and Anderson (synthesized by Ehrlich and Bertheim; D.R.P. 213,155 (1907) to Hoechst) |
| 1932 | 'Atebrin' . . | Malaria . . . . . | Kikuth (biology); Mauss and Mietzsch (chemistry) |
| 1932 | 'Mapharside' . | Syphilis . . . . . | Tatum and Cooper (synthesized by Ehrlich and Bertheim; D.R.P. 235,391 (1908) to Hoechst) |
| 1935 | 'Prontosil'. . | Systemic bacterial infections . | Domagk (synthesized by Mietzsch and Klarer, D.R.P. 607,537 (1932)) |
| 1935 | Sulphanilamide . | Systemic bacterial infections . | Tréfouël, Tréfouël, Nitti and Bovet (synthesized by Gelmo, 1908) |
| 1935 | 'Acaprin' . | Babesiasis (cattle) . . . | Kikuth (synthesized by Schönhöfer and Henecka) |
| 1937 | 'Bayer 7602' . ('Chagavlon') . | {Chaga's Disease (Tryp. cruzi)} . . . | Mazza, Cossio and Zuccardi (synthesized by Iensch) |
| 1938–43 | Phenanthridines. | Trypanosomiasis (cattle) . . | Browning, Morgan, Robb and Walls; Carmichael and Bell (synthesized by Walls) |
| 1938 | Sulphapyridine . | Systemic bacterial infections . | Whitby (biology); (synthesized by Ewins, Phillips and Newberry, B.P. 516,288) |

* Of little or no value in man.

† These discoveries were in the nature of consolidation and extension of earlier work.

| *Date* | *Drug* | *Condition in which used* | *By whom introduced* |
|---|---|---|---|
| 1940 | Sulphadiazine | Systemic bacterial infections | Feinstone, Williams, Wolff, Huntington and Crossley (biology); (synthesized by Roblin, Williams, Winnek and English) |
| 1940 | Sulphaguanidine | Bacillary dysentery | Marshall, Bratton, White and Litchfield (synthesized by Buttle, Dewing *et al.*, 1938) |
| 1938–42 | Di-amidines | Trypanosomiasis, Leishmaniasis, Babesiasis | King, Lourie and Yorke (synthesized by Ewins, Ashley, Barber, Newbery and Self); Adler and Tchernomoretz (Leishmaniasis) |
| 1941 | Penicillin | Systemic bacterial infections* | Florey, Abraham, Chain, Fletcher, Gardner, Heatley, Jennings, Orr-Ewing and Sanders (based on preliminary work by Fleming, 1929) |
| 1942 | 5-Aminoacridine (aminacrine) | Chronic sepsis of wounds | Poate (clinical trials); Rubbo and Maxwell (biology); Albert and Goldacre (chemistry) |
| 1943 | 4:4′-Diaminodiphenylsulphone and *derivatives* | Leprosy | Faget, Pogge *et al.* (synthesized by Fromm and Wittmann, 1908) |
| 1945 | 'Paludrine' | Malaria | Fairley (clinical trials); Davey (biology) (synthesized by Curd and Rose) |
| 1945 | Streptomycin | Tuberculosis | Waksman and Schatz (biology) and staff of Mayo Clinic |
| 1947 | 'Chloromycetin' | Typhus | Smadel and Jackson |

It is convenient to take 1899 as the start of Ehrlich's ntense interest in chemotherapy. This is the year in which ie was appointed Director of the Institute for Experimental Therapy in Frankfort. He was then forty-five years old, quite late in life to be founding a new branch of learning! n Frankfort, Ehrlich was brought face to face with the German synthetic chemical industry and at the Hoechst works, he saw the manufacture of a profusion of synthetic analgesics, antipyretics and anaesthetics.* It seemed logical o him that, as it was obviously possible to synthesize substances of low molecular weight that could differentiate between various cells of man, it should be equally possible o synthesize other small molecules which would differen- iate between man and his parasites. The emphasis on low molecular weight illustrates the contrast which Ehrlich always made between immunotherapy (i.e. therapy with vaccines and sera) and chemotherapy. He realized that mmunotherapy is a matter of strengthening the defence forces of the body, but he conceived of chemotherapy as a direct attack upon the parasite. The problem, as he saw t, was to find chemicals with very much stronger affinities or the parasites than for the tissues of the host.

It is not surprising that Ehrlich should have initiated his branch of selective toxicity for he had spent the previous twenty years on studying the selective affinity of issues. First he had studied the distribution of lead between the different tissues of the mammalian body; then he distribution of dyes. The latter subject led him to make ome outstanding studies in histological staining, and his nethod for differentially staining white blood cells is still he best known. His studies of dyes also led him to iscover the differing oxygen-affinities of tissues and to

* Examples of such substances are the *analgesics* (i) phenacetin (Hinsberg and ast, 1887), (ii) Antipyrine or phenazone (Knorr, 1889), (iii) pyramidon or midopyrine' (Knorr and Stolz, 1896); the *analgesics and antirheumatics*, sodium salicylate introduced into medicine by Buss in 1875, (ii) aspirin, rst used on man by Dreser in 1899; the *hypnotics*, (i) chloral hydrate (Liebrich, 69), (ii) paraldehyde (Cervello, 1882), (iii) barbitone or 'Veronal' (Fischer and n Mering, 1903); the *local anaesthetics*, e.g. procaine or 'Novocain' (Einhorn, 05).

grade them by the ability to reduce one or all of a series of dyes with different reduction potentials (methylene blue, indophenol, alizarin blue).

The extraordinary selectivity shown by an antibody for the corresponding antigen then engaged his attention. Many of the more important immunological techniques that are used to-day were discovered by Ehrlich in this period. It is interesting to recall Ehrlich's interpretation of the immune process, viz. that an antigen* has two distinct groups or regions, viz. the *haptophore* (anchorer) and the *toxophile* (poisoner).† Mammalian cells, he believed, have 'side-chains' which contain *receptors*, i.e. groups or regions that are complementary to the haptophores, and hence anchor them. This combination, he taught, was in itself harmless but brought the toxophile close enough to the cell to poison it. The normal function of the receptors was to anchor nutrient molecules.

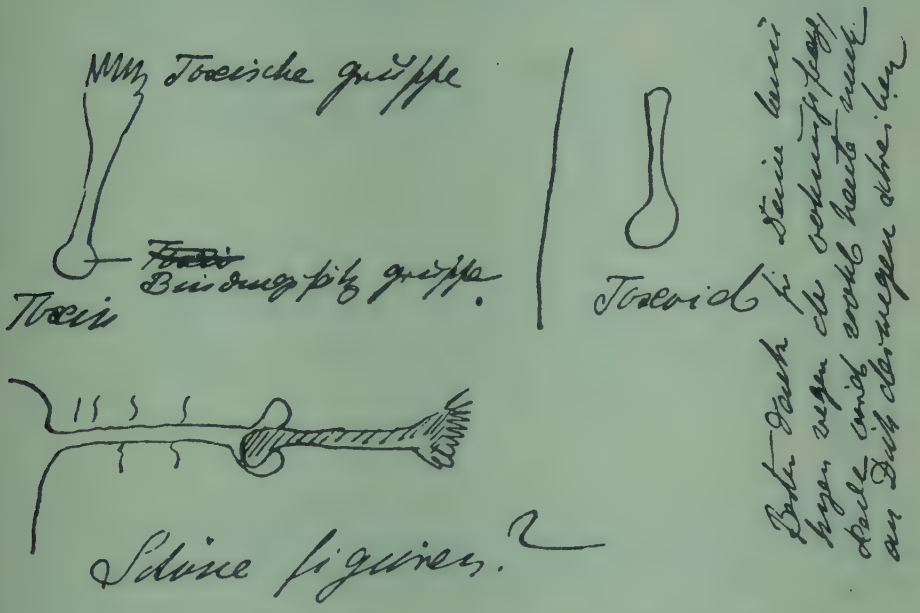
Ehrlich applied this part of his immunological hypothesis to explain the mode of action of chemotherapeutic agents. They too were supposed to have distinct haptophoric and toxophilic groups (this is now known to be true for a great many drugs) and to combine with cellular receptors whose normal function was to take part in cellular nutrition or respiration. (A further part of the immunological hypothesis which dealt with the manufacture of antibody through the shedding of side-chains was not taken over into the chemotherapeutic hypothesis and is no longer regarded as correct by immunologists.)

It may be interesting to look at some of the symbols which Ehrlich coined to illustrate these ideas, the immunological in Fig. 1 and the chemotherapeutic in Fig. 2. In the immunological symbols, the suction-cups and

* The *antigen* is the toxic protein (derived e.g. from bacteria) to which certain cells of the mammalian body respond, more or less successfully, by producing a complementary protein, the *antibody* which is discharged into the blood-stream and combines specifically with this particular antigen (but no other) to form an innocuous product.

† Toxoids have intact haptophores, but the toxophiles have been blocked, e.g. with formaldehyde.

ntacles suggest something macromolecular, like folds in protein molecule. The chemotherapeutic symbols, on ne other hand, took on a precise chemical character, e.g. ne example of an (imaginary) aldehyde group in a cellular olecule combining with a hydrazine group in a drug. hrlich never tired of saying that drugs act upon cells by rdinary chemical reactions. This is substantially the



g. 1—Ehrlich's explanation of immunochemistry in his own symbols (taken from a letter to Carl Weigert, 1898, in which he gives the first pictorial representation of his side-chain theory).

esent-day view, although to us the phrase 'ordinary emical reactions' conjures up a much wider variety of nds than was known in Ehrlich's time.

Ehrlich's concepts of chemically reactive groups on drugs d of chemically reactive receptors for them on cells nstituted an enormous advance in biological thought. s concept that the effective drugs would have a fairly v molecular weight has been substantiated, as will be thered from Table 2, which shows a representative ection of chemotherapeutic drugs in use to-day. The lecular weights range from 170 to 1500, the latter being

unusually high for a really active substance. On the other hand, γ-globulin, of which antibodies are fashioned, has a molecular weight of 200,000.

In 1904 Ehrlich cured trypanosomiasis in mice with trypan red, which thereby became the first man-made chemotherapeutic agent.* This aroused widespread interest but unfortunately the drug was inactive in man. In 1906, the *Georg Speyer Haus* was built in Frankfort for

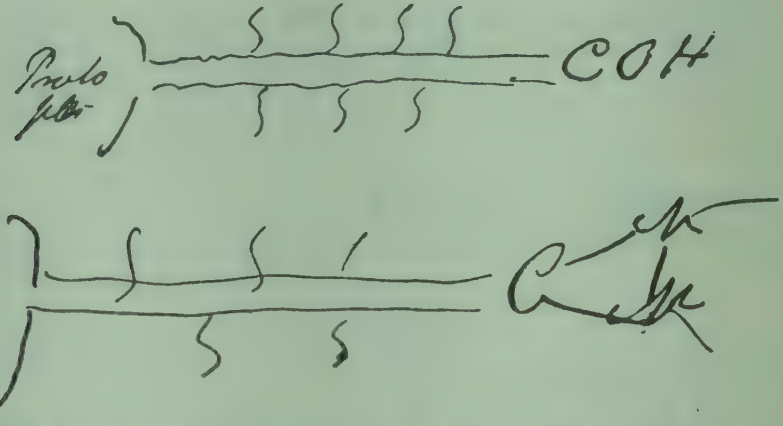


Fig. 2—Ehrlich's explanation of chemotherapy in his own symbols. A metabolically important molecule in a cell is imagined as having an aldehyde group that normally anchors nutritional intermediates. In the lower drawing, this ability is lost because the aldehyde group has been blocked by combination with an imaginary drug containing a diamine group.

Ehrlich so that he could devote all his time to chemotherapy. Meanwhile, Breinl and Thomas, in Liverpool, had shown that an arsenical drug, 'Atoxyl', had a favourable action on human trypanosomiasis and this discovery influenced Ehrlich to commence prolonged experimentation with aromatic arsenicals.

It may come as a surprise to some that Ehrlich's name figures so little in the list of new drugs (Table 1); indeed, he discovered only one remedy of immediate clinical usefulness, viz. 'Salvarsan'. The reason is that Ehrlich

* Two years earlier, Laveran and Mesnil had used arsenious acid in the trypanosomiasis of mice. All the mice died, but they 'died cured' and this was considered an important advance.

TABLE 2

THE LOW ORDER OF COMPLEXITY OF TYPICAL CHEMOTHERAPEUTIC AGENTS

| *Substance* | *Number of atoms per molecule* | *Molecular weight* |
|---|---|---|
| Mapharside' | 16 | 199 |
| ulphanilamide | 19 | 172 |
| Chiniofon | 22 | 351 |
| Aminacrine | 25 | 194 |
| ulphadiazine | 27 | 250 |
| Chloromycetin | 32 | 323 |
| Paludrine' | 33 | 253 |
| tilbamidine | 36 | 264 |
| Neoarsphenamine | 36 | 466 |
| enicillin | 41 | 334 |
| Quinine | 48 | 325 |
| Atebrin' | 58 | 400 |
| Emetine | 75 | 480 |
| treptomycin | 77 | 579 |
| uramin ('Bayer 205') | 126 | 1,428 |
| *or comparison* | | |
| Glucose | 24 | 180 |
| $\gamma$-Globulin | 30,000 | 200,000 |

as, at heart, a scientist, one who realized that there would few advances in the technology of his subject until some the laws governing it had been discovered. In the case 'Salvarsan', which he introduced in 1910, just five years ter Schaudinn demonstrated that *Treponema pallidum* s the causative organism of syphilis, Ehrlich spent ecious years in finding the right dosage scheme for his w drug. He had hopes, at first, of curing infections with single large dose; but experience was to show that the ly possible course was to administer such doses as the tient could easily stand, spread over a period of several nths.

It might be thought that the work done by Ehrlich and his small band of collaborators in the Georg Speyer Haus would stand high in the esteem of all contemporary scientists. On the contrary, Ehrlich's vivid and picturesque language made enemies as well as friends. One has only to read his address to the German Chemical Society (Ehrlich, 1909) to encounter a surprising degree of rhetoric, dogmatism and reliance on intuition rather than logic. Many people of this period thought that his interpretations had quite outrun the experimental evidence.

Chief among Ehrlich's critics was Uhlenhuth, an influential pathologist with some quite sound experimental work to his credit. Uhlenhuth contended that drugs had no direct action on the parasite, but worked by stimulating the natural resistance of the host. Although forced to yield to the evidence that drugs did not produce antibodies in the host's blood, Uhlenhuth maintained his ideas about indirect action in a less specific way. His ideas commanded a great deal of respect, and even as late as 1937 Levaditi had no compunction in stating that sulphonamides probably acted by increasing phagocytosis.

Uhlenhuth made the most of the fact that 'Atoxyl' and trypan red cure trypanosomiasis and yet do not attack trypanosomes in the test-tube (Uhlenhuth, 1907). This objection was taken seriously because these substances were two of Ehrlich's most important experimental tools. But in 1909 Ehrlich discovered that trivalent arsenicals were trypanocidal in the test-tube and he suggested that pentavalent arsenicals would also be found active if it were possible to keep trypanosomes alive in culture until they had time to reduce the drug. That this was actually so, was not proved until fifteen years after his death. However, Ehrlich did show that pentavalent arsenicals became active *in vitro* if they were first incubated with animal tissues (e.g. liver), and this served to explain their activity *in vivo*.

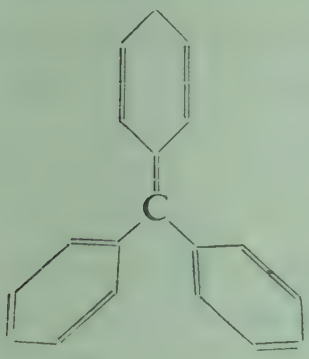
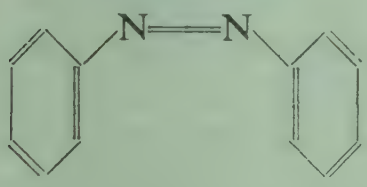
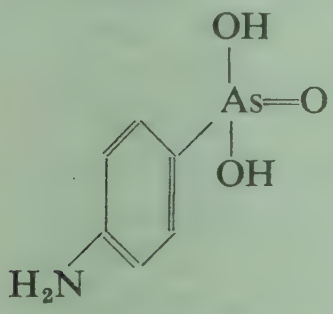
Meanwhile Levaditi (1908) had tried to reconcile the views of Ehrlich and Uhlenhuth by postulating that drugs

uld act chemically on the parasite, but only after they d been chemically altered by the host. He referred rticularly to the effect of atoxyl on syphilis. We recog-ze to-day that this hypothesis is true in a few cases (for ample, 'Atoxyl' and 'Prontosil') and untrue in many hers (e.g. 'Mapharside' and sulphanilamide).

In 1907, Franke and Roehl, who were working with rlich, made a discovery which has tremendously ad-nced our understanding of how chemotherapeutic agents t. This discovery was the phenomenon of *drug resistance* hich arose in the following way. When a mouse suffering om trypanosomiasis was treated with doses that were too all to cure, a relapse took place, and the renewal of eatment with the drug eventually failed because the panosomes developed a resistance which was hereditary d usually irreversible. As Yorke has since observed, sistant strains may require up to 250 times the normal ncentration of drug before they are injured. Such assive dosage is usually more than the host can tolerate. Ehrlich encountered several distinct types of resistance trypanosomes. Parasites which had become resistant to pan red resisted all other azo dyes. Other strains, which re resistant to 'Atoxyl', resisted all other phenylarsonic ds, and a third type, resistant to parafuchsin, resisted other triphenylmethanes. Yet a strain resistant to one these three classes of drugs did not resist the other two sses unless specially trained to do so. Here at last was dence of chemical specificity on the part of the parasite! These three types of resistance are illustrated in Table 3. Ehrlich noticed that trypanosomes that were resistant to pan red did not absorb it, whereas susceptible trypano-nes were stained red by the drug. The first step in the ion of trypan red on trypanosomes was clearly the mical combination between some chemical group on dye and a chemical group on the parasite. The resistant asites, whether by selective breeding or in some other , had lost the group that combined with the dye.

TABLE 3

MAIN TYPES OF DRUG RESISTANCE IN TRYPANOSOMES

| *Type* | *Example* |
|---|---|
| Triphenylmethane  | Parafuchsin |
| Azobenzene  | Trypan red |
| Aromatic arsenicals  | 'Atoxyl' |

Later, Ehrlich recognized two classes of arsenical resistance between which there was no cross-resistance, and to-day we recognize three classes.* This evidence, plus the

* Membership of these classes is not connected with the state of oxidation of the arsenic, but depends on the nature of substituents in the benzene ring (Chapter VII).

ıct that trypanosomes do not become resistant to inorganic rsenicals, offers proof that the groups which cause aroma- c arsenicals to be taken up by the parasites are not the rsenoxide groups responsible for the eventual death of the arasite. Plainly a haptophoric group is present as well as toxophilic one in the selectively toxic arsenicals.

After the clinical introduction of 'Salvarsan' in 1910 (as reat a triumph for Ehrlich's chemist Bertheim as for the naster himself) Ehrlich lived for only five more years. Ouring this period two more drugs were discovered that vere active in mice but not clinically successful in patients Optochin' and 'Trypaflavin'). Rogers confirmed that metine was the active principle of ipecacuanha in amoebi- sis. Vianna demonstrated the value of antimonials in eishmaniasis, and Browning, a pupil of Ehrlich, discovered ne very first chemotherapeutic antibacterials, acriflavine nd proflavine. Apart from an extensive use of these cridines, the First World War passed without any notable dvance in chemotherapy. The systemic control of bacterial nfection by chemotherapy was still an unrealized dream, nd the feeling was widespread that chemotherapy had romised more than it had given or was likely to give. his pessimism was remarkable, for Ehrlich had initiated hemotherapy simply because he felt that the possibilities f immunotherapy were exhausted.

Ehrlich's name, after his death, continued to be honoured -in fact, more so than when he was a thorn in the side of ertain of his contemporaries; but it was rather as the great mmunologist and pathologist than as the father of chemo- herapy. One might almost say that not until 1935, when omagk demonstrated the control of systemic bacterial fections with 'Prontosil', did chemotherapy recover its rmer position in the eyes of the medical world. What a aradox! For Domagk's work, although systematic, was mpirical in conception, whilst the years that preceded it ere packed full of scientific discoveries which vindicated d extended Ehrlich's hypotheses.

In 1920, two drugs were discovered which have become the basis of our present-day treatment of sleeping sickness. These are tryparsamide, a pentavalent arsenical derived from, but less toxic than, the optimistically named 'Atoxyl', and 'Bayer 205', a colourless and highly effective analogue of trypan red. 'Bayer 205' is also used for prophylaxis (a single dose gives immunity for three months). The next notable advance was the introduction of 'Plasmoquine' and 'Atebrin' (1926, 1932), the first synthetic antimalarials.

All these discoveries were made as the result of pursuing clues which Ehrlich and his school had revealed. For example, the synthetic antimalarials grew out of knowledge of the antimalarial action of methylene blue (Guttmann and Ehrlich, 1891), but this investigation had been held up, awaiting the discovery of a suitably infected laboratory animal on which to carry out the tests. Once Roehl seized upon the idea of using birds for this purpose, progress was rapid. There are many other examples of the arrest of chemotherapeutic progress until a development is made in some other branch of science. The most surprising feature of this period was the introduction of two valuable arsenicals, 'Carbarsone' (for amoebiasis) and 'Mapharside' (for syphilis). Both these drugs had been discovered by Ehrlich and his colleagues and used by them in experimental work; but these workers had not foreseen their clinical application. Indeed, until the discovery by Laidlaw, Dobell and Bishop in 1928 of a method of cultivating entamoebae *in vitro*, the experimental study of amoebicides had been severely handicapped.

In the very next year a first-rate discovery was made when Yorke, Adams and Murgatroyd (1929) found out how to keep trypanosomes alive and unmetamorphosed in the test-tube for one to two days at a time (Ehrlich had to be content with moribund parasites which died in the control-tubes almost as fast as in those containing drugs).

This new technique of the Liverpool school seemed

kely to lead to a better understanding of the manner in vhich drugs acted on trypanosomes. Within two years, Yorke, Murgatroyd and Hawking (1931) showed that when ormal trypanosomes were treated with a solution of a rivalent arsenical, they were rapidly killed and the solution vould not kill fresh trypanosomes. On the other hand, vhen arsenic-resistant trypanosomes were treated with a rivalent arsenical, they were not killed, and the residual olution would still kill susceptible trypanosomes. They ound it possible even to titrate the trypanosome against he arsenical and thus calculate the number of 'chemo-eptors' on its surface. It will be recalled that Ehrlich had hown that trypanosomes were stained by the dye trypan ed in the test-tube, but not if they were resistant. Unfor-unately trypan red does not kill trypanosomes during their rief life *in vitro*, so that Ehrlich's results had not convinced ceptics. On the other hand, Yorke's results showed very lainly that at least one type of drug resistance was caused y a lack of appropriate chemical groups in the resistant arasite, and that susceptible strains of parasite possessed roups with which they fixed the drug and so removed it rom solution.

It remained but to translate this circumstantial evidence nto direct evidence by showing that the bodies of the killed arasites contained measurable amounts of arsenic and hat the resistant parasites contained none. This was done 1932 by Reiner, Leonard and Chao. Advances in tech-ique made it possible for other workers to show that xation of drugs by the parasite could take place while the tter was actually circulating in the host's blood-stream. on Jancsó (1932) used the fluorescent microscope to show at 'Trypaflavin' was taken up in this way, and Singer and ischl (1934) and Fischl, Kotrba and Singer (1934) demon-rated that this happened with antimony, gold and arsenic. hese workers also demonstrated similar effects on irochaetes and plasmodia.

Significant as these discoveries were, they were by no

means the only ones of fundamental importance in this period. Ehrlich had already found that, even when dealing with drugs that were effective both *in vitro* and *in vivo*, small doses were more effective in the living animal than in the test-tube. He attributed this enhancement to co-operation with the natural defence forces of the body, which would cope with the parasite once the drug had disorganized the latter's metabolism. It remained for Kritschewsky (1927–1928) to show that the host's reticulo-endothelial system provided such co-operation, because when this was artificially damaged, the efficiency of chemotherapeutic drugs was considerably lowered.

A new aspect of chemotherapeutic action was revealed through the discovery of a totally unsuspected phenomenon, therapeutic interference (Browning and Gulbransen, 1922). As an example we may consider the case of a mouse infected with trypanosomes. If a small dose of a dye called parafuchsin is injected, the parasites are stained red by it, and they are not killed by the subsequent injection of powerful trypanocidal agents. It is as though the parafuchsin becomes attached to the parasite in such a way that it physically covers up the receptors appropriate for the other drugs—receptors which had been shown by drug-resistance experiments to differ from those binding parafuchsin (see also Chapter VII).

From Ehrlich's concept of the separateness of haptophoric from toxophilic portions of a drug, it follows that a parasite should not necessarily be harmed by merely taking up a foreign substance, and that it may have to be capable of reacting further with it, presumably using other chemical groups for this purpose, before injury occurs. Further evidence along these lines was provided by Fischl and Singer (1935), who showed that, in the host, malarial parasites and trypanosomes took up three acridines, 'Atebrin', 'Trypaflavin' and 'Rivanol', but that the malarial parasites were harmed only by the 'Atebrin', and the trypanosomes only by the 'Trypaflavin' (see Table 4.)

TABLE 4

DEMONSTRATION THAT MERE UPTAKE DOES NOT ENSURE TOXICITY

| *Organism* | *Substance* | *Taken up* | *Toxic* |
|---|---|---|---|
| Trypanosomes | 'Rivanol' | + | − |
| | 'Atebrin' | + | − |
| | 'Trypaflavine' | + | + |
| Plasmodia | 'Rivanol' | + | − |
| | 'Atebrin' | + | + |
| | 'Trypaflavine' | + | − |

As momentous as any discovery in the period we are considering was the first demonstration of the exact chemical nature of the union between a drug and a parasite. This was accomplished by Voegtlin and his colleagues in the United States Public Health Services, who showed that the toxic action of arsenicals was due to the formation of As—S bonds with essential thiol groups in the parasites (see Chapter III).

Thus we come to the end of this period (1919–1935) with a feeling of satisfaction that the scientific principles of chemotherapy, foreshadowed by Ehrlich, had actually been established. It is now generally agreed that substances of relatively low molecular weight make the most satisfactory chemotherapeutic drugs, that their action is directly on the parasite,* that their reaction with the parasite is chemical in nature and that there are two distinct reacting groups in many of the most active drugs.

As the result of testing more than a thousand azo-dyes in a search for antistreptococcal drugs which would be active in the blood-stream, Domagk (1935) discovered

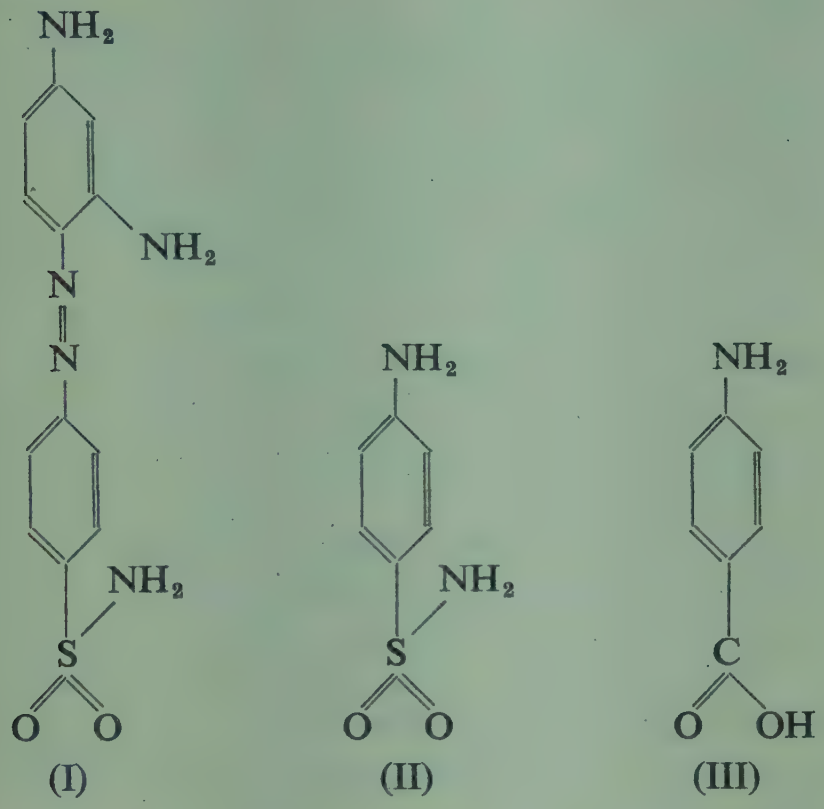
* Two apparent examples of indirect action of chemotherapeutic agents are (*a*) diethylcarbamyl-4-methylpiperazine ('Hetrazan'), which is thought to combine with microfilaria in a way that makes them more readily destroyed by the host (Hawking, Sewell and Thurston, 1950) and (*b*) streptomycin and aureomycin which are thought to repress amoebiasis by killing bacteria which produce growth-factors essential for the amoebae.

'Prontosil' (I), whose outstanding clinical possibilities in puerperal septicaemia were first explored in England by Colebrook and Kenny (1936). This highly utilitarian discovery did more than the two decades of brilliant scientific work which preceded it to revive interest in chemotherapy, an interest which has not waned since. Actually, 'Prontosil' was not an end, but a beginning as far as sulphonamides were concerned. A discovery of great importance was made later in 1935 by Tréfouël and his co-workers at the Pasteur Institute, who found that the inactivity of 'Prontosil' towards bacteria in test-tube experiments could be overcome by the addition of a reducing substance. In devising this experiment they were guided by Ehrlich's work on the activation of pentavalent arsenicals. Later, they were able to show that not only did the body reduce 'Prontosil' to give sulphanilamide (II), but that this substance was equally active in the treatment of bacteriaemia. Had the French workers not made this discovery, the evolution of sulphonamides would have proceeded on the 'hit and miss' lines that had been so typical of the research efforts of the drug industry. Sulphapyridine, sulphadiazine and sulphaguanidine would probably not yet be known, and immense resources would have been squandered in the search for new types of *coloured* sulphonamides.

An important scientific advance was made when Marshall (1937) showed that the effect of sulphanilamide was proportional to the concentration reached in the blood of the patient being treated, and that, for a given dose, this varied from patient to patient. This was the beginning of the bioanalytical control of blood-levels during chemotherapy.

Fleming in 1940 showed that the antibacterial action of sulphonamides was abolished by certain kinds of organic matter, principally peptone, tissue extracts and pus. Woods (1940) was able to obtain a crude extract, from yeast, which reacted as an aromatic amino-acid and specifically antagonized the antibacterial action of sulphanilamide. By trying

n turn all sorts of aromatic amino-acids, Woods found nat *p*-aminobenzoic acid (III) was the most effective. elbie, in the same year, showed that this acid would revent sulphanilamide from exerting its curative effect on nfected mice.




(I) (II) (III)

Nevertheless, at that time, *p*-aminobenzoic acid was not known for certain to occur in nature. Later in the same year (1940), Rubbo and Gillespie isolated the acid, chemically pure, from yeast, and what is more, they showed that it was a growth-factor for a bacterium with which they were then working; that is to say, the bacterium would not grow without it, just as a human being cannot survive without vitamins.

A few months previously Fildes had made a brief but most impressive appeal for a more rational approach to chemotherapy. He pointed out that the action of a drug on a parasite had much in common with the inhibition of an enzyme by a foreign substance and that many of the

most successful enzyme inhibitors were substrate-analogues. (This position was already fairly well understood in pharmacology.) Fildes discussed two main types of enzyme-inactivation: (i) the drug combines with the enzyme directly, or (ii) the drug combines with the substrate of an enzyme.

Fildes had been led to these thoughts partly by Voegtlin's work on arsenic, partly by his own work on mercury, and in particular by the biological antagonism between sulphanilamide (II) and *p*-aminobenzoic acid (III). He postulated that this acid was a normal metabolite for the bacteria, and that sulphanilamide (because of its close steric similarity and because of the similar chemical nature of the two ends of the molecule) deceived the enzyme into accepting the drug instead of the metabolite, resulting in a disruption of bacterial metabolism. As so often happens with enzyme inhibition, this is a reversible reaction, and unless a great excess of sulphanilamide is maintained around the bacterium, the *p*-aminobenzoic acid will replace the sulphanilamide on its relevant enzyme, and metabolic activity will be restored. As uncombined *p*-aminobenzoic acid does not seem to have a function in the human body, it is easy to see how sulphonamides can be so selective in their toxic action.

This visualization of some of Ehrlich's receptors as enzymes marked a big advance in the scientific development of chemotherapy. Here was an added incentive for workers to study the internal and external enzymes of parasites, to discover the nature of their metabolites and to synthesize molecules that would be like enough to the metabolites to be accepted by the bacterium, and yet unlike enough to interrupt its vital processes. The analogy has often been made that enzymes and their metabolites have a lock-and-key relationship; but a chemotherapeutic agent should be a key that fits the lock only well enough to jam it (Chapter III).

No account of the recent history of chemotherapy would

be complete without a reference to the antibiotics, i.e. toxic substances of fairly low molecular weight secreted by bacteria and moulds. The clinical possibilities of these substances were visualized by Florey in 1938, and he and his talented school have investigated many examples and made possible, in 1941, the clinical utilization of penicillin, which Fleming had discovered in 1929. The outstanding success of penicillin in local and systemic infections, a success attributable to its having a toxicity so low that it must be ranked as one of the most chemotherapeutic of all known drugs, has led to the investigation of innumerable other antibiotics.* In the vast majority of cases the substances have proved to be too toxic for clinical use, which is hardly surprising in view of the fact that they had not been devised for man's use. There is no doubt that the further study of antibiotics will bring to light much of biochemical interest and, from time to time, some very valuable drugs of the calibre of streptomycin, aureomycin and chloromycetin. The name 'antibiotic' is probably a misnomer as there is no evidence that organisms secrete these substances to fight their natural enemies. Indeed, if penicillin were of survival value to *penicillium notatum*, the few strains which manufacture this substance would be the commonest examples of this species whereas, in nature, they are actually most uncommon.

Among the most promising new trends in chemotherapy, mention must be made of the interest now being taken in the architecture of the cell, as expressed in chemical terms (cf. Sponsler and Bath, 1942). Thanks largely to the pioneer work of Pauling, we now know the bond-angles, bond-lengths and bond-strengths of all the various combinations which can take place between atoms, and we can form a clear idea in our minds of the sizes of biologically important molecules, such as drugs and co-enzymes, relative to the size of living cells (see Frontispiece). We should

* Antibiotics of low clinical potency, such as acetic acid, alcohol and hydrogen peroxide, have been used medicinally from time immemorial, usually in crude forms.

have no trouble in calculating that there is room for 2000 fully extended protein molecules (of average length 1000 A) in the exterior surface of a streptococcus. With the aid of Hirschfelder atomic models we can build ourselves very good representations of simple molecules on the scale of one centimetre to the Ångström unit. For more complex

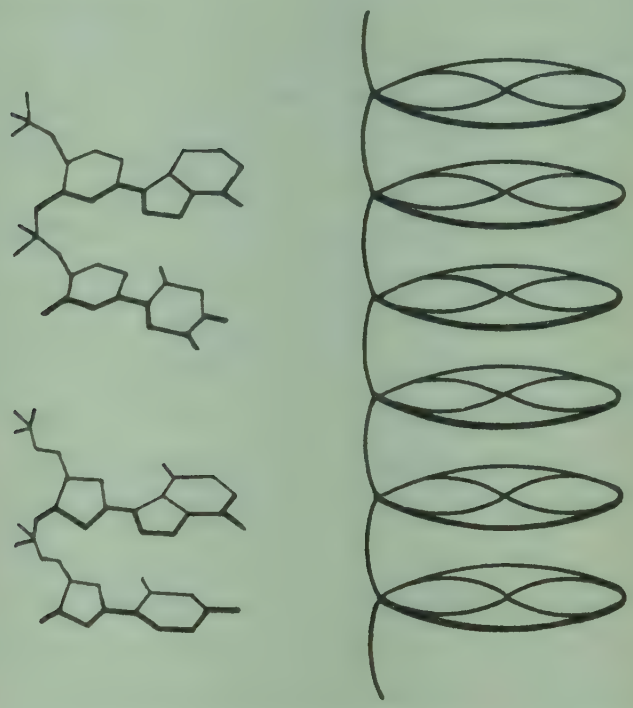
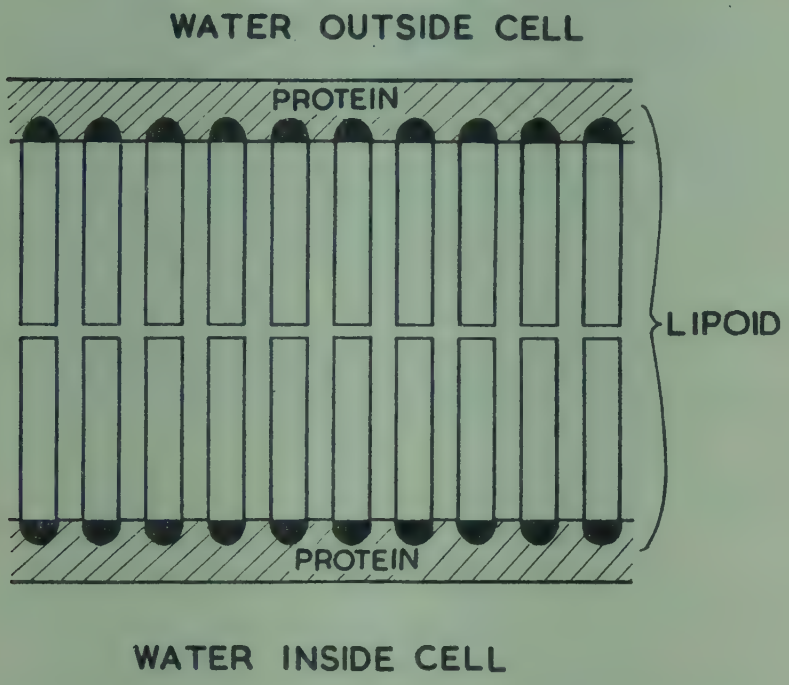


Fig. 3—Diagrammatic representation (right) of the column of nucleotides (left) constituting the unit of deoxyribonucleic acid. Each nucleotide is 3·3 A from the next.

molecules, such as the proteins, working models can readily be built, to the same scale, out of 'Perspex'. The dimensions of such a model are furnished by the three principal repeating distances (10 A, 7 A and 4·5 A), each at right angles to the others, the polypeptide backbone being represented by the perspex, and the side-chains by pins. Such a model should be capable of folding and unfolding.

A useful picture (Fig. 3) of the arrangement of the atoms in nucleic acid has been made by Astbury and Bell (1938) and was discussed further by Astbury (1947).

Knowledge of the structure of complex cell-components ch as these has led to working hypotheses of the arrange- ent of atoms in other important cell-components. Of ese we may instance the attempted construction of the tive patch on succinic dehydrogenase (Chapter III, rmula VI) and the ideas of Davson and Danielli (1943) the nature of the cell-membrane. The cell-membrane




g. 4—Diagrammatic representation of cell-membrane, after Davson and Danielli (1943).

of special interest in drug studies because of its highly lective permeability. Much that is known about its ucture and function can be turned to practical account. e majority of cell-membranes, but not all, behave as ough they were a layer of lipoids, two molecules thick th a monomolecular protein layer on either side (as own in Fig. 4). Organic ions penetrate rather poorly less provided with lipophilic groups. On the other hand, utral molecules with fewer than 12 carbon atoms pene- te well. However, every additional water-attracting up (in excess of one) slows penetration, so that most

cells are only very slowly permeable to glycerol and no cells are permeable to sugars by simple diffusion. Sugars require an energy-consuming mechanism to pump them into the cell (cf. also glutamic acid in Chapter VII).

Another important current trend in chemotherapy is the attempt to place clinical research on a more scientific basis. Too often it has been found that a series of potential drugs which have been graded into one order, by virtue of tests on laboratory animals, have had to be placed in a quite different order when tested on a statistically significant number of patients under conditions as rigorously controlled as in the laboratory. Volunteers for studies of this kind are not always available, and the most has to be made of the material presented by hospital wards. But the valuable results obtained by Fairley and his co-workers from 1,000 healthy men, voluntarily infected with malaria, show what can be done under ideal conditions (Fairley, 1946).

## *CHAPTER SEVEN*

# SOME SPECIAL ASPECTS OF CHEMOTHERAPY. SULPHONAMIDES, PENICILLIN, DRUG-RESISTANCE, THERAPEUTIC INTERFERENCE, ADSORPTION

IN the last chapter we traced the discovery of the underlying principles of chemotherapy. Before leaving this subject, the opportunity will be taken of dealing with certain sections of it which deserve more detailed treatment than they have so far been accorded.

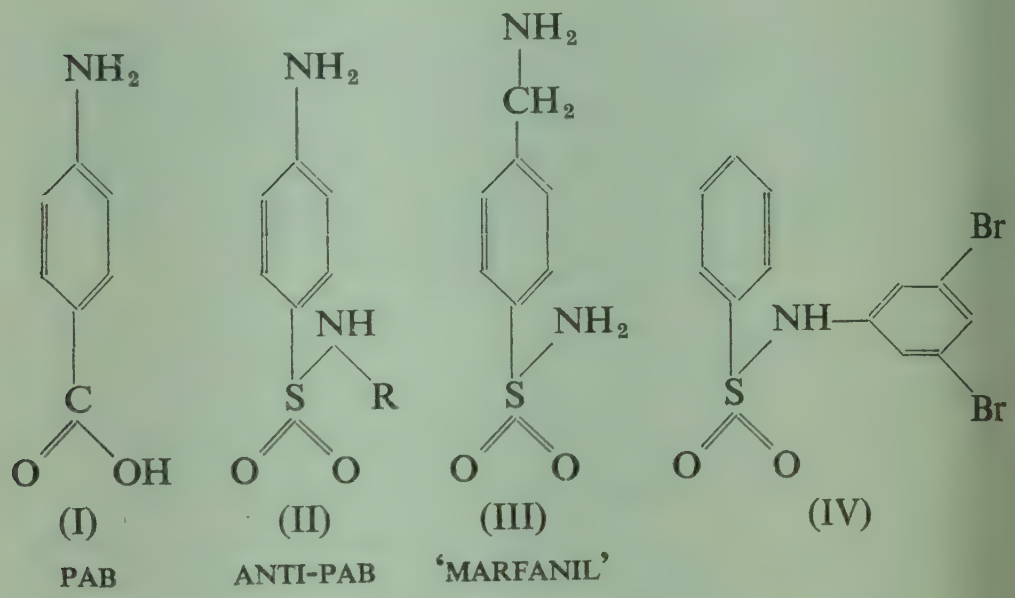
### (i) *Sulphonamides*

We cannot begin to discuss sulphonamides without at once running into a difficulty of nomenclature because there are at least three distinct classes of antibacterial substances having a sulphonamido-group as a substituent in the molecule. In every-day speech, 'the sulphonamides' comprise that series of useful drugs which work by competing with *p*-aminobenzoic acid (I). Common examples of this group are sulphanilamide, sulphadiazine, sulphathiazole, sulphapyridine and sulphaguanidine, and they are represented by formula (II).

Another class of sulphonamide is represented by 'maranil' (III) which has a strongly ionized basic group thanks to the non-conducting paraffinic group ($-CH_2-$) which separates the amino-group ($-NH_2$) from the base-weakening electronic field of the nucleus. In this it differs from the first class of sulphonamides, all members of which have amino-groups incapable of ionizing at pH 7. 'Maranil' has only a paper resemblance to sulphanilamide (II, R=H) and is not antagonized by *p*-aminobenzoic acid. It apparently acts simply as a kationic antibacterial and, as would be expected, is antagonized by hydrogen ions.

Compared with the heteroaromatic kationic antibacterials, described in Chapter IV, its action is feeble.

The third class of sulphonamides, represented by formula (IV), lacks the *p*-amino group typical of the first class, but has as its R-group a nucleus with strongly electron-attracting substituents. Members of this class are not antagonized by *p*-aminobenzoic acid (Goetchius and

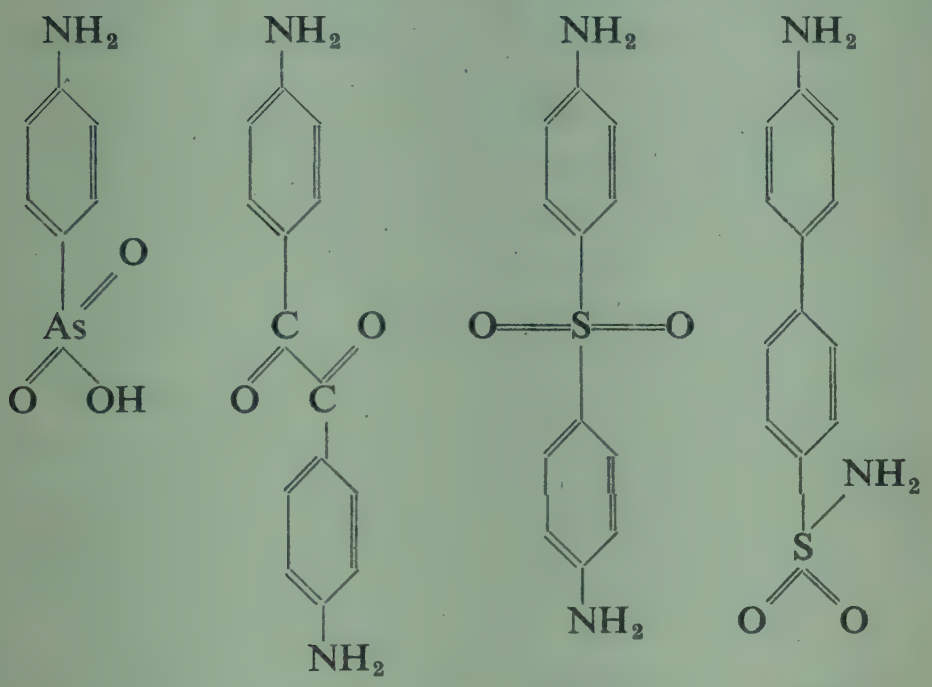



Lawrence, 1945; Schmidt and Sesler, 1946). No $pK_a$ values for them have been recorded, but they appear to be well-ionized acids and may turn out to be functioning as simple anionic antibacterials, like dodecane sulphonic acid, in which case they will be antagonized by hydroxyl ions (cf. Chapter IV). No member of this class has been found sufficiently active to be given a place in clinical medicine. If an amino-group is inserted in the same position as in sulphanilamide, substances are obtained whose action is partly antagonized by *p*-aminobenzoic acid.

Obviously a special name is required for those sulphonamides which compete with *p*-aminobenzoic acid (PAB). To coin new names unnecessarily is undesirable, but here the need is acute. I suggest that these substances be called

*ti-pabs*. This name is not musical to the ear, but it is f-explanatory. Let us use it until someone suggests mething better.

All anti-pabs, be it noted, are not sulphonamides and me of them do not contain any sulphur at all. All that needed is that the molecules of these metabolite analogues ould have a sufficient (but not too great) degree of




(V) (VI) (VII) (VIII)

emblance to the molecule of *p*-aminobenzoic acid. The ertion of a methyl-group into the 2- or 3-positions of AB gives intermediate types which have neither pab nor ti-pab activity. However, the insertion of a chlorine m into either the 2- or 3-positions of PAB makes an ive anti-pab (Wyss, Rubin and Strandskov, 1943).

*p*-Aminobenzenearsonic acid ('atoxyl' V) is another ti-pab. In general, arsonic acids are not antibacterial Albert, Falk and Rubbo, 1944), but this substance forms an exception. The reason is that it is the only enical which resembles *p*-aminobenzoic acid sufficiently l to be able to compete with it (Hirsch, 1942). Another

sulphur-free anti-pab is diaminobenzil (VI). This substance is several times more active than sulphanilamide but slightly inferior to sulphathiazole (Kuhn, Möller and Wendt, 1943). Even the sulphur-containing anti-pabs need not have a sulphonamide group, as *p*:*p*′-diaminodiphenyl sulphone (VII) shows. This sulphone is many times more active than sulphanilamide,* but is correspondingly more toxic unless the amino-groups are blocked in such a way that the drug is slowly liberated through hydrolysis.†

What features are required in a molecule for it to have an anti-pab action? Firstly, it is essential that it should have a primary aromatic amino-group. This must not be substituted in any way, unless by a group which will readily break down in the body and liberate the primary amino-group. An azo- or anil-linkage can be depended on to break in this way (as in 'prontosil' and 'soluseptasine'), but acyl- and alkyl-groups will not do so. Hence *p*-methylaminobenzene-sulphonamide, the secondary amine corresponding to sulphanilamide, has no antibacterial action, even *in vivo*. Secondly, an electronegatively charged group is required, placed *para* to the amino-group, and at the same distance as in *p*-aminobenzoic acid. The electronegativity is usually supplied by an electron-attracting non-ionized group such as C=O or S=O. One interpretation that can be put upon Bell and Roblin's curve (Chapter IV, Fig. 14) is that the electronegativity is better supplied by an anion, although such a group is electron-repelling.‡ However, the fact that those of Bell and Roblin's compounds which were 50 per cent ionized at pH 7 were most active may mean simply this: the

* e.g. 16 times as active against pneumococcal infections of mice (Litchfield, White and Marshall, 1940).

† 'Promin', 'Diasone' and 'Sulphetrone' are as (VII) but with the amino-group loosely blocked in various ways, with this end in view.

‡ The negative charge due to an electron-attracting group is quite small, although it can be increased somewhat by the presence of electron-repelling groups in the same molecule. A molecule with a fractional charge of this kind could be held by a receptor bearing a positive ionic charge, but not by one having only a fractional positive charge. On the other hand, the negative charge due to an anion is large, but invariable in its size. A molecule with an ionic charge could be held even by a receptor with a fractional positive charge.

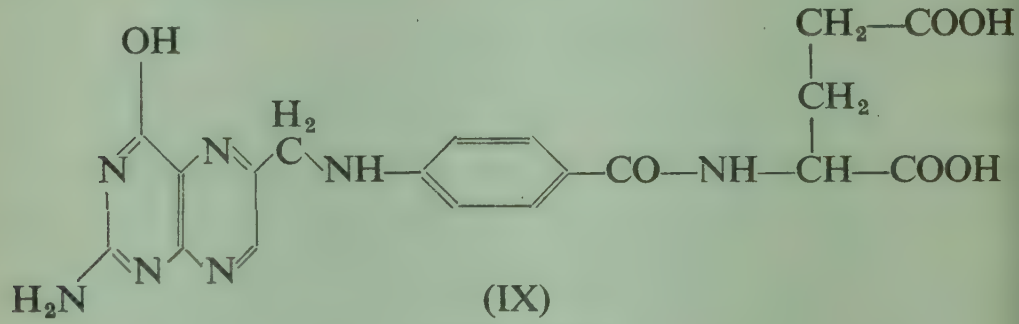
electronic distribution in the molecule which favours the formation of anions, also increases the size of the fractional negative charge on the non-ionized molecules simultaneously present. This explanation allows of the non-ionized molecules being the active form of *all* anti-pab substances. In the original paper by Bell and Roblin (1942) both possibilities were canvassed, but in more recent studies, the latter explanation has been favoured (Roblin, 1946). The necessity for keeping the distances between the amino- and the electronegative-groups similar to that obtaining in *p*-aminobenzoic acid is illustrated by 4-amino-4′-sulphonamido-diphenyl (VIII), which has no anti-pab effect (Kumler and Halverstadt, 1941*).

Sevag (1946) attempted to show that the antagonism between anti-pab drugs and PAB was a case of competition between two entirely artificial substances similar to that between morphine and allyl*nor*morphine (Chapter III). Sevag argued at some length that PAB was not a normal constituent of *pathogenic* organisms, but this, together with other ingenious but ill-founded objections, have been capably answered in the light of new knowledge by Woods (1947, cf. Work and Work, 1948).

The discovery, in 1946, that *p*-aminobenzoic acid forms part of the molecule of folic acid (IX) opened up new approaches to the study of the action of anti-pab drugs. With some bacteria, e.g. *Strept. faecalis* Ralston and *Lactobacillus arabinosus* (known on the Continent as *Strept. plantarum*) there is evidence to suggest that all of the *p*-aminobenzoic acid is used (perhaps rather inefficiently) to make folic acid. In Table 1, for example, it can be seen that there is an exact proportionality between

* Fox and Rose (1942) and Schmelkes *et al.* (1942) held that the biological activity was the exclusive property of sulphonamide *anions*, which is palpably untrue (cf. sulphaguanidine which forms no anions). Bell and Roblin (1942) thought the distances between the oxygens in the anions of PAB and the sulphonamides were practically identical and that this was relevant, but Kumler and Daniels (1943) showed that there was a substantial difference. The latter authors stated that biological activity in sulphonamides runs parallel to the flattening effect on the molecules produced by increasing dipole moments as the substituents were varied. This notion was disproved by Bell and Roblin (1944).

the amount of sulphadiazine required to inhibit the former organism and the amount of *p*-aminobenzoic acid required to reverse this inhibition. On the contrary, the amount of folic acid required for reversal is constant, regardless of the amount of sulphadiazine used. This indicates that the sulphadiazine interferes with the synthesis of folic acid from *p*-aminobenzoic acid, but does not interfere with the utilization of folic acid itself. Thus, the addition of folic acid can sidestep the competition. Similarly, *L. arabinosus* (which requires an external source of PAB) is inhibited by sulphonamides and this inhibition is reversed competitively by PAB and non-competitively by folic acid. The amount of folic acid* produced by this organism is ordinarily proportional to the amount of PAB in the medium. However, if sulphanilamide is also present, it is reduced in proportion to the amount used, over a 10,000-fold range of sulphanilamide concentrations (Nimmo-Smith and Woods, 1948; Nimmo-Smith, Lascelles and Woods, 1948). The production of folic acid is inhibited in *B. coli*, also, by sulphanilamide (Miller, 1944).




(IX)
Folic acid

It will be observed in Table 1 that thymine (X), which is a pyrimidine occurring in nucleic acid, can also antagonize the action of sulphadiazine on the streptococcus and that it does this somewhat non-competitively. It is not surprising that so large an amount is needed, because thymine is not a catalyst (like PAB and folic acid) but a

* Measured by disintegration of the bacteria, and titration of the homogenized culture against *L. casei* which responds to folic acid but not to PAB.

## TABLE 1

### TYPES OF INHIBITION

| *Substance* | *Amount needed for 50% antagonism of effect of Sulphadiazine on* Strept. faecalis *Ralston* | | | |
|---|---|---|---|---|
| SULPHADIAZINE . . . . . | 1 | 10 | 100 | 1000 |
| *p*-Aminobenzoic acid . . . . | 0·003 | 0·03 | 0·3 | 3·0 |
| Pteroylglutamic acid (Folic acid) . . | 0·0003 | 0·0003 | — | 0·0003 |
| Thymine . . . . . | 0·06 | 0·25 | 0·25 | 0·25 |

All values are μg. per ml.

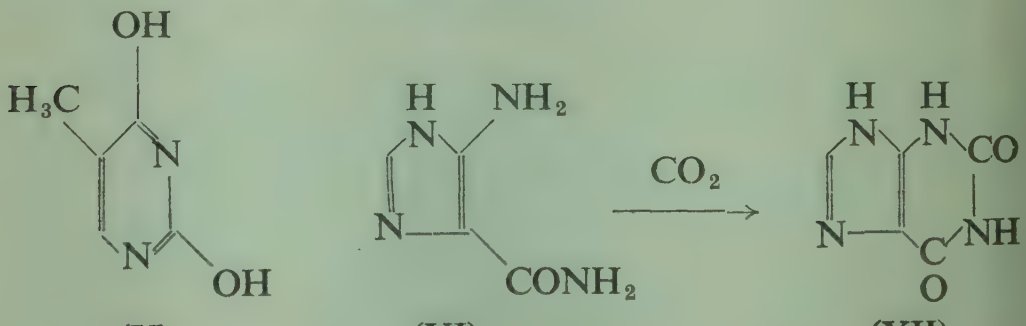
Lampen and Jones (1946).

cell-constituent, the demand for which will increase as the cell continues to grow.

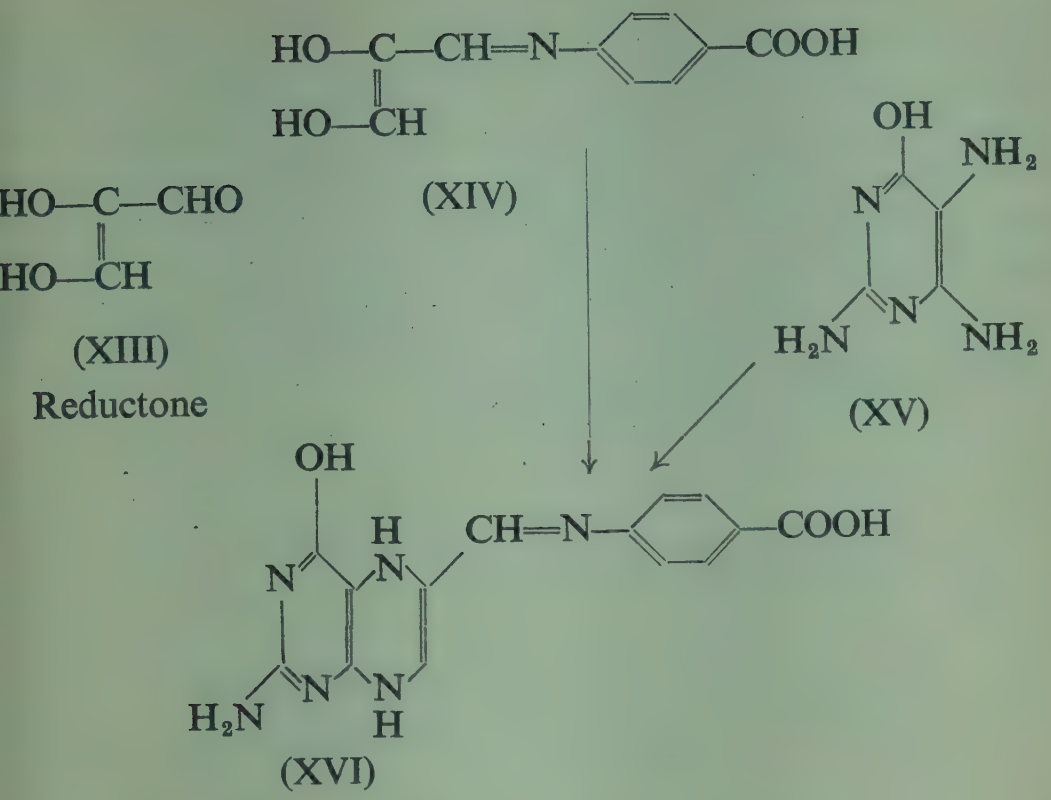
With other species, similar non-competitive antagonisms by purines have been observed, and it is believed that one of the principal functions of folic acid may be to act as a co-enzyme in the synthesis of various purines and pyrimidines required for nucleic acids (Stokes, 1944; Lampen and Jones, 1947).

The action of anti-pab drugs on some bacteria is not reversed by folic acid, e.g. in the mutant strain of *B. coli* requiring PAB for growth. Either these organisms are impermeable to folic acid, or they use PAB to make other important substances besides folic acid. In some organisms very small concentrations of anti-pab drugs can be reversed by methionine. 2-Chloro-4-aminobenzoic acid quite specifically antagonizes that part of anti-pab action which is reversed by this amino-acid. From kinetic studies on *B. coli*, Shive and Roberts (1946) conclude that PAB is concerned in the synthesis of methionine through some other pathway than the intervention of folic acid.

Concerning the synthesis of purines by folic acid, it is interesting to note that 4-aminoiminazole-5-carboxamide (XI) accumulates in culture media where organisms have been treated with insufficient sulphanilamide to inhibit them (Stetten and Fox, 1945; Shive *et al.*, 1947). This substance, on paper, requires only carbon dioxide to become xanthine (XII), a purine which is readily convertible in the cell to other purines.




(X) Thymine (XI) (XII) Xanthine

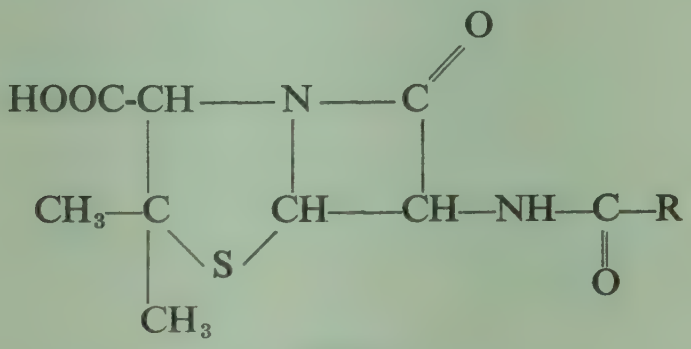



The first step by which PAB is converted to folic acid appears to be through union between PAB and the widely distributed aldehyde known as reductone (XIII) (Forrest and Walker, 1948). The resulting anil (XIV) has actually been isolated from the medium in which yeast was growing (Bell, Cocker and O'Meara, 1948). That reductone was somehow concerned in the action of anti-pab drugs was first suggested by O'Meara, McNally and Nelson (1944). Forrest and Walker (1948) have shown that the anil (XIV) readily unites with a simple pyrimidine (XV) to give pteroic acid (XVI, shown in a tautomeric form). Pteroic acid is folic acid (IX) minus the glutamic acid moiety. The pyrimidine (XV) has not yet been identified in nature.

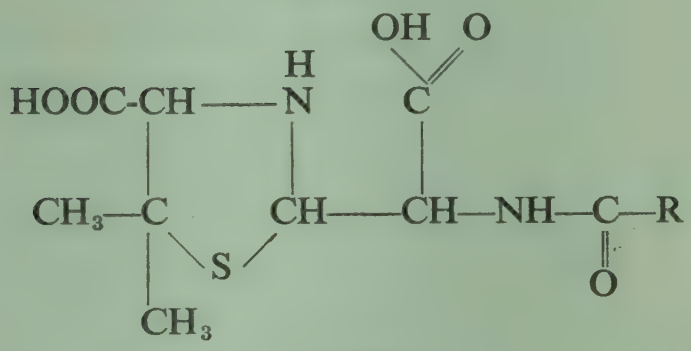
(ii) *Penicillin*

Pencillin is a generic name for a series of closely related substances (XVII) produced by strains of the mould *Penicillium notatum*. Penicillin K is distinguished by a comparatively poor therapeutic action (Tompsett, Shultz

and McDermott, 1947). This defect has been traced by Davis and Dubos (1947) to the heptyl side-chain which would give it, alone of the penicillins, a strong tendency to enter the lipophilic interiors of molecules of albumin in the blood-stream. In penicillins F and G, the side-chains are, respectively, too short and too bulky for loss of activity to occur in this way. Manufacturers try to keep their products free from penicillin K. Most of the crystalline penicillin sold is the G type.



(XVII)



(XVIII)

Penicilloic acids

Penicillin G (R=benzyl)
Penicillin F (R=$\triangle^2$-pentenyl)
Penicillin K (R=heptyl)

The penicillins are all readily hydrolysed to the corresponding penicilloic acids (XVIII) by alkali, cupric ions or the specific enzyme penicillinase. Penicilloic acid is not antibacterial and no way is known of reversing the hydrolysis.

Gale has shown that the antibacterial properties of penicillin run parallel to the ability of this drug to prevent the passage of amino-acids into the bacterial cell from the outside environment. With the apparently sole exception of lysine, amino-acids do not diffuse freely through the cell-membranes of those organisms most susceptible to penicillin (the majority of these organisms are Gram-positive). It is known that glutamic acid, for example, is pumped in by an energy-consuming process. Penicillin halts this inverse-secretion of amino-acids, although the utilization of glutamic acid within the cell remains unaffected so long as the internal supply lasts. It will be seen from Fig. 1 that, soon after the addition of a little penicillin to a growing culture of *Staph. aureus*, the accumulation of glutamic acid is halted. Coincidentally, growth ceases (Gale and Taylor, 1947).

Gale was able to discover a great deal more about this phenomenon by making use of drug-resistance techniques. When staphylococci were grown in the presence of increasing amounts of penicillin, in the course of many generations, all the bacteria were killed except some natural mutants which were insensitive to penicillin and hence became selected for survival. Some of these mutants were found to tolerate 600,000 times as much penicillin as the normal strain. Such mutants were found to have the ability to synthesize their own requirements of amino-acids from ammonia and glucose so that they were in no way incommoded by the blocking action of penicillin on amino-acid uptake. Throughout this process of adaptation, the penicillin resistance of the organism developed in proportion as the ability to synthesize amino-acids was acquired. Moreover, the concentration of penicillin required to inhibit assimilation at any stage was always the same as that required to inhibit growth (Gale and Rodwell, 1949).

In another series of experiments, staphylococci were trained to do without the majority of the fifteen amino-acids that they normally require from their medium. This was

done by Gladstone's technique of culturing the bacteria in ammonia-glucose solutions which became progressively poorer in amino-acids until only cysteine and histidine had to be supplied externally. At this stage, it was found that

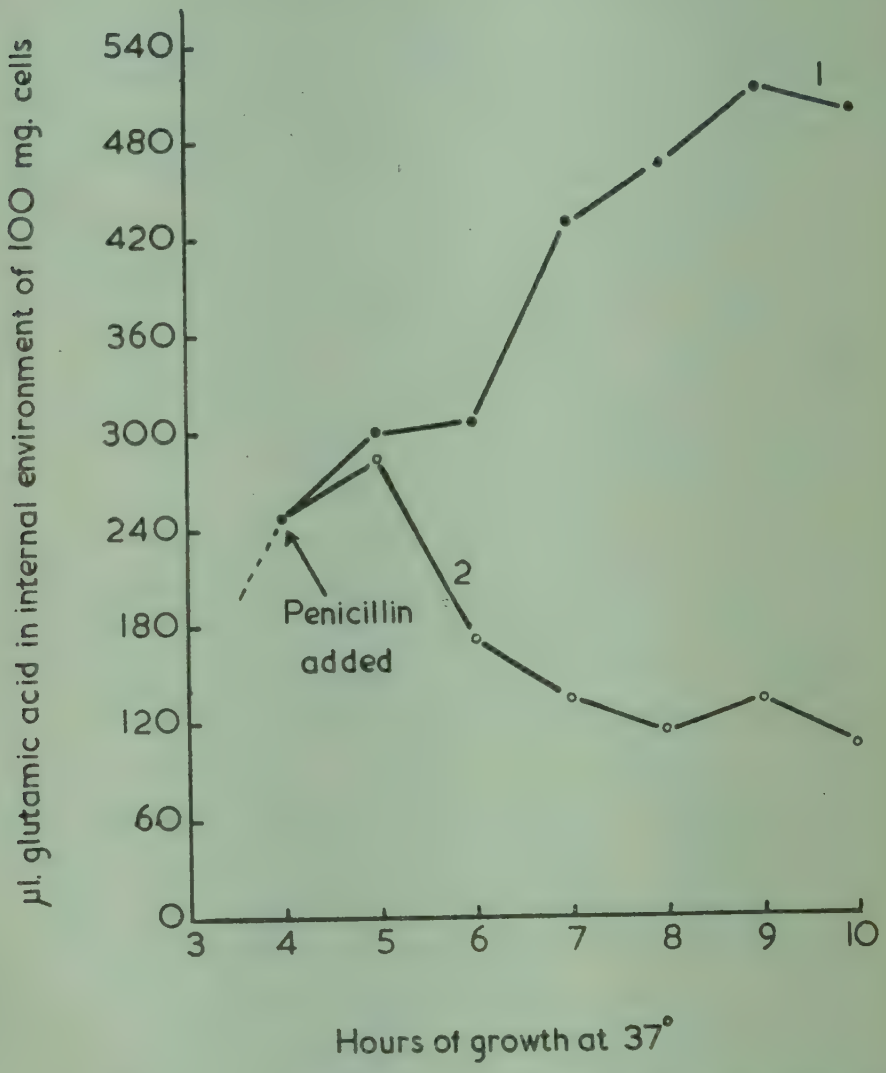



Fig. 1—Effect of penicillin (0·002 mg. per mil) on the accumulation of glutamic acid by growing cells of *Staph. aureus*. *Curve* 1: no penicillin; *curve* 2: penicillin added after $3\frac{1}{2}$ hours. Medium: casein digest, glucose and Marmite (*Gale and Taylor*, 1947).

the mutants selected by this process were 5,000 times as resistant to penicillin as the original strain, although they had never previously been in contact with penicillin (Gale and Rodwell, 1949).

Against this coherent mass of reasoning stands the fact hat *B. subtilis*, a Gram-positive organism, remains very ensitive to penicillin even when grown in a medium in vhich the sole source of nitrogen is furnished by ammonium alts (Hunter and Baker, 1949; Grelet, 1949).

Gram-negative rods, which are not very sensitive to penicillin, normally synthesize their requirements of amino-cids. However, a mutant of *Salmonella typhimurium* which required an external source of cysteine, was several times more sensitive to penicillin than the normal strain (Plough and Grimm, 1949).

It is not yet known how penicillin blocks the amino-acid assimilating system. The prevention of the uptake of amino-acids may be only the end-result of a more fundamental reaction inhibited by penicillin. It is known that the uptake of penicillin by the cell can be prevented by prior treatment with acetic anhydride (Rowley *et al.*, 1950). Now, the formula of penicillin (XVII) suggests that it might act as a powerful, if rather specific, acylating agent. It is known to combine with hydroxylamine and also with a number of 1 : 2-aminothiols,* by acylation, its hydrolysis to penicilloic acid being the corresponding acylation of water. Rinderknecht (1946) has suggested that it may combine with a cysteine side-chain of some special protein. However that may be, it is known that penicillin inhibits the enzyme responsible for the hydrolysis and resynthesis of mononucleotides (Macheboeuf, 1948; Mitchell, 1949), a process believed to be concerned with energized uptake. Moreover, nucleic acid is said to end the inhibition induced by penicillin in *Staph. aureus* even when added three hours later (Pandalai and George, 1947; Faguet, 1948). Conversely, penicillin causes this organism to shed ribose-nucleic acid (Pratt and Dufrenoy, 1949).

Whatever the chemical process may prove to be, there s no doubt it is very specific. Studies with radio-active

* High concentrations of such substances can inhibit the antibacterial effect f penicillin, but this probably has no biological significance.

penicillin (using $S^{35}$) have shown that as little as 10 molecules of penicillin per cell (*Staph. aureus*) are strongly inhibitory (Rowley, Cooper, Roberts, and Lester-Smith, 1950). The penicillin was found to be very firmly held by the cells, but a similar experiment showed that 740 additional molecules per cell are loosely bound (Maass and Johnson, 1949). Resistant strains, even when not penicillinase producers, do not take up this radio-active penicillin.

It has been shown that chloramphenicol (chloromycetin) and aureomycin inhibit protein synthesis in bacteria when present in bacteriostatic concentrations (Gale and Paine, 1950). Streptomycin has no such effect.

(iii) *Drug resistance*

In Chapter VI we have already discussed Ehrlich's three classes of trypanocidal substances, as defined by drug-resistance studies. These were the azo-benzenes, the triphenylmethanes and the arsenicals. Organisms made resistant to a member of any one class, automatically became resistant to any other member of that class, but not to members of other classes. The arsenical class, however, is now known not to be homogenous, but to consist of three sub-classes. The first of these is typified by (XIX) and is distinguished by the presence of water-attracting substituents (e.g. $-OH$ or $-NH_2$) such as do not ionize as anions. Nearly all the arsenical drugs in common use belong to this sub-class because members of it are distinguished often by a favourable therapeutic index. It has also been found that the highly kationic acridine known as trypaflavine or euflavine (XXII) belongs to this sub-class because trypanosomes made resistant to it are automatically resistant to arsenicals of type (XIX).

The second sub-class of arsenicals (XX) is distinguished by having substituents which lack strong hydrophilic properties (e.g. $-CH_3$ and $-NO_2$). Trypanosomes which are resistant to the other two sub-classes of arsenicals are

ll susceptible to this. However, this sub-class holds no erapeutic interest, as the toxicity to mammals is as high the toxicity to the parasites.

The third sub-class (XXI) is distinguished by substi-ents which are ionized as anions at pH 7, such as the rboxylic acid groups. Such substances are usually inno-ious to the host, but also to the parasite. However, one ample, *p*-arsenosophenyl-butyric acid has been found otent enough for clinical use and has proved valuable in ses of resistance to members of the first sub-class.

In these three sub-classes of arsenicals, the state of idation of the arsenic is of no consequence. These facts, ken in conjunction with the fact that no resistance to senious acid has ever been achieved, although sedulously ught, show quite plainly that the non-arsenical part of e molecule is responsible for the uptake of the drug by e parasite, although it is known that the arsenoxide oup is responsible for the eventual toxic action of the ug (see Chapter III). Hence there are at least three stinct mechanisms for the uptake of aromatic arsenicals, d these would seem to be mutally exclusive (King and rangeways, 1942). Thus we see how a study of drug-sistance phenomena can contribute to knowledge of the ode of action of drugs.


(XIX)

(—OH, —As : O, $CONH_2$ or $SO_2NH_2$ may replace $-NH_2$)


(XX)

(H—, $NO_2$— or $-OCH_3$ may replace $-CH_3$)


(XXI)

(—$SO_3H$ may replace —COOH)

As far as is known, resistance in trypanosomes always takes the same form: the parasite so modifies the chemistry of its surface that the drug is no longer taken up but remains behind in the medium. On the other hand, a susceptible trypanosome can accumulate in its interior a 500 times greater concentration of arsenic than exists in the external medium (Eagle, 1945). Some organisms, such as the spirochaete of syphilis, do not seem to be capable of evolving an arsenical resistance. Many bacteria create resistance to drugs in other ways than by limiting uptake.

One way in which bacteria can become resistant is by secreting a destructive enzyme. Although *Staph. aureus* becomes resistant to penicillin in the test-tube by achieving independence of the environment as a source of amino-acids (see above), the commonest strain of penicillin-resistant organisms isolated from patients manages to survive by secreting penicillinase which hydrolyses the drug to inert penicilloic acid (cf. Barber, 1947). The two penicillin-resistant strains of staphylococci are serologically distinct.

Evidence is accumulating that staphylococci can destroy certain aminoacridines by oxidizing them, e.g. with a peroxidase (Selbie, Albert and Simon, unpublished). Anti-bacterial acridines can be divided into two classes: (i) those which resist oxidation by light and air and also by iron-catalysed hydrogen peroxide and (ii) those that are susceptible to these oxidizing agents (which apparently operate by making free hydroxy-radicals). Class (i) consists mainly of 5-aminoacridine and its derivatives, and no resistance to members of this class can be induced. Class (ii) consists mainly of derivatives of 2-aminoacridine and it is to members of this class that the bacteria become resistant.

Another type of resistance in bacteria takes the form of secreting an excessive amount of the substance to which the drug is a metabolite-antagonist. Several workers, for instance, have shown that staphylococci, pneumococci and

gonococci become resistant to the usual concentrations of sulphonamide drugs by secreting extra quantities of *p*-aminobenzoic acid (cf. Landy and Gerstung, 1944; Landy *et al.*, 1943). The *p*-aminobenzoic acid produced in this way has now actually been isolated chromatographically (Lemberg, private communication). On the other hand, *B. coli* can be trained to resist low concentrations of sulphathiazole without producing *p*-aminobenzoic acid, although, when trained to resist higher concentrations, extra *p*-aminobenzoic acid is actually produced (Lemberg *et al.*, 1948). The existence of two distinct resistance mechanisms are thus revealed just as with *Staph. aureus* and penicillin.

All the above forms of resistance are most likely to be explained by the survival of mutant strains which are naturally resistant but which form only a minute part of the original inoculum. The death of the susceptible organisms provides the opportunity for these mutants to proliferate and to mutate further. Demerec (1945) has shown how penicillin-resisting mutants could arise in this way.

If the concentration of a drug is increased, there is a chance that a mutant will be present that can resist the adverse metabolic effects of this new concentration, but the likelihood of this happening time after time is very slender. However, one or two cases have come to light where a drug seems to be able directly to accelerate the rate of mutation.

Quite a different resistance mechanism has been discovered by Hinshelwood (1946) in studying the effect of proflavine on the Gram-negative rod, *B. lactis aerogenes*. This is a process of continuous variation in which the organism becomes adapted to growing in precisely that concentration of antibacterial to which it has been trained. As with other forms of drug resistance, this effect is transmitted to all succeeding generations. An example is given in Fig. 2.

Hinshelwood considers that the bacterial cell has two

distinct sets of enzymes, one for growth and one for division, a hypothesis which is agreeable to many biologists. He next assumes that the substances which cause adaptation act by blocking the enzyme(s) responsible for division.

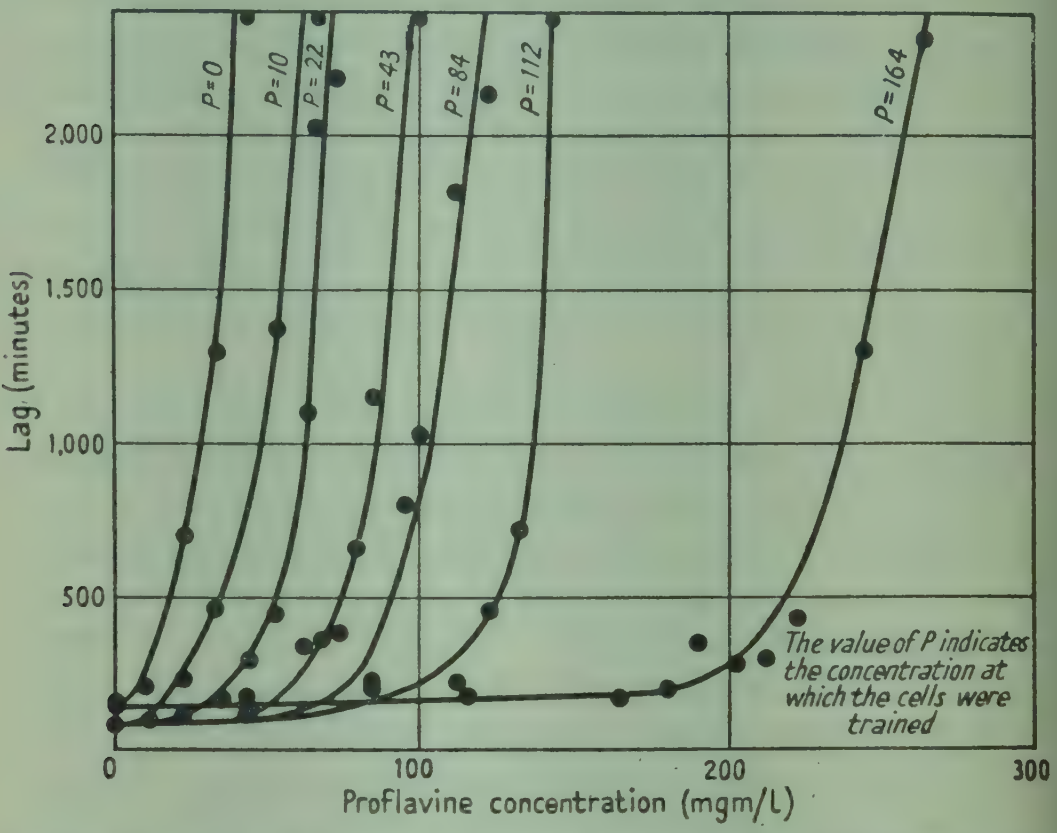



Fig. 2—Family of curves showing that *B. lactis aerogenes* can grow in any concentration of proflavine to which it has been previously trained, but not in any stronger concentration (*Hinshelwood*, 1946).

The organism, unable to divide, goes on growing and increases in size while at the same time continuing to produce growth and division enzymes in the old proportions. As soon as it has produced more of the division enzyme than there is drug to block, division takes place. The two halves of the organism are each longer than is usual, and when they grow and the time comes for them to divide, they will each have a greater quantity of division enzyme than the drug can block, provided the former concentration of the drug is not exceeded.

Studies of this kind have been carried out with various acridines, with methylene blue, crystal violet and various sulphonamides. Hinshelwood agrees that mutation can play a leading part in the resistance phenomena of other drugs and other organisms, but argues that even in such cases selection could not show the graded response which is often obtained, hence the type of adaptation illustrated in Fig. 2 may often be superimposed upon a lower degree of resistance due to mutation.

(iv) *Therapeutic interference*

The name *therapeutic interference* was coined by Browning and Gulbransen (1922) to describe the following phenomenon which they discovered. Mice suffering from trypanosomiasis could not be cured by injecting the usual dose of euflavine (XXII) if they had previously been fed on parafuchsin (XXIII). Actually, there was a complicating factor in this experiment, because parafuchsin is itself slightly trypanocidal; however, this difficulty was overcome by using a sufficiently small dose. A typical experiment of this kind is shown in Table 2. It should be noted that organisms cannot pass the interference effect on to the next generation, a point of distinction from drug-resistance phenomena.

TABLE 2

THERAPEUTIC INTERFERENCE—INJECTIONS INTO TRYPANOSOME-INFECTED MICE

| *Parafuchsin* | *Euflavine* | *Result* |
|---|---|---|
| Nil | Nil | Died 5th to 7th day (parasites +++) |
| 0·05 | Nil | Died 5th to 7th day (parasites +++) |
| 0·25 | Nil | Died 5th to 7th day (parasites +++) |
| Nil | 0·5 | Cured on 3rd day |
| 0·05 | 0·5 | Died 6th to 7th day (parasites +++) |
| 0·25 | 0·5 | Died 7th day (parasites +++) |

Schnitzer (1926).

It can be seen from this table that 1 part of parafuchsin can prevent 10 parts of euflavine from acting. Interference between these two substances has also been demonstrated *in vitro*, the parafuchsin preventing the euflavine from killing the organism (v. Jancsó, 1931). The same antagonism has also been demonstrated respirometrically with yeast (Wright and Hirschfelder, 1930) and with trypanosomes (Scheff and Hasskó, 1936). A typical example is shown in Table 3. It is interesting to notice that both substances separately depress glucose consumption, but that a mixture of both substances has no such effect.

TABLE 3

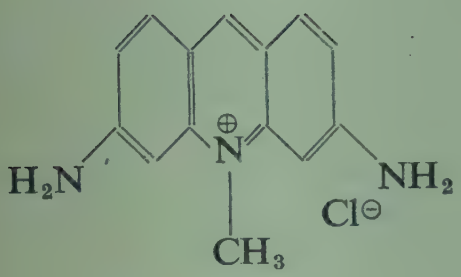
PARAFUCHSIN *v.* EUFLAVINE ON TRYPANOSOMES IN RAT-SERUM

| *Rat previously injected with* | *Oxygen consumed per* $10^6$ *tryps. in* $5\frac{1}{2}$ *hours cmm.* | *Glucose consumed per* $10^6$ *tryps. in* $5\frac{1}{2}$ *hours* $\mu g$. |
|---|---|---|
| Control | 30·0 | 20·0 |
| Parafuchsin (4·9 mg.) | 23·0 | 11·6 |
| Euflavine (6·5 mg.) | 20·5 | 8·0 |
| Parafuchsin (5·6 mg.) + Euflavine (6·6 mg.) | 26·4 | 20·4 |

Scheff and Hasskó (1936).

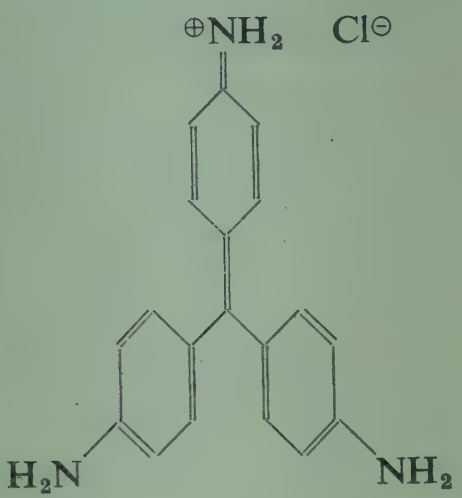
The term therapeutic interference has been confined to cases where there is considerable chemical similarity between the two substances and hence no obvious likelihood of their reacting chemically with one another. They thus appear to form pairs, like the metabolites and their metabolite analogues discussed in Chapter III, the principal

difference being that in therapeutic interference, neither substance occurs naturally. A case of therapeutic interference in pharmacology has already been mentioned, viz. the interference by allyl*nor*morphine with the action of morphine in dogs (Unna, 1943).



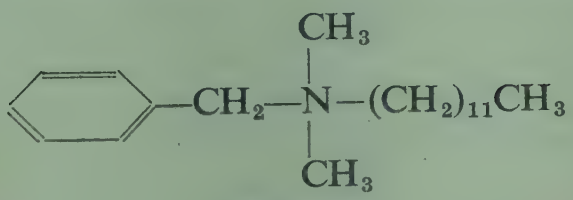
(XXII)

Euflavine (Trypaflavine)



(XXIII)

Parafuchsin



(XXIV)

The explanation of such cases of therapeutic interference has generally been along these lines: the less biologically active substance has a greater affinity for a receptor on the cell than the more active substance has. Yet Hasskó (1935) carried out some colorimetric analyses which show that the affinity of trypanosomes for parafuchsin is very small compared with their affinity for euflavine.* As the

* It is just possible that the difficulties of working colorimetically with these dyes was not realized, and hence the correct conditions for converting the colourless carbinols and ethyl ethers back to the coloured ions may not have been imposed (cf. Goldacre and Phillips, 1949).

adsorption of both substances would follow the mass action law, the above explanation must be incorrect.

Two alternative explanations have recently been put forward, tentatively (Albert, 1949). One hypothesis is that the parafuchsin and the euflavine are each adsorbed by a different kind of receptor and that both kinds of receptor occur close together. As parafuchsin is comparatively inert, it would appear that it becomes attached to a receptor that has no very vital part to play in the parasite's metabolism. The inference is that parafuchsin interferes with the action of acriflavine by sterically obstructing it from combining with a receptor that is vitally important to the parasite. There is yet little evidence to test this hypothesis.

The other hypothesis was suggested by some results which Valko and Dubois (1944) obtained when working with dodecyl trimethyl benzyl ammonium chloride (XXIV). They found that the bactericidal concentration of this substance had to be doubled if one-quarter of its weight of a higher homologue (hexadecyl trimethyl benzyl ammonium chloride) was added. It seems to the writer that this is a clear case of the formation of a mixed micelle.* If we may make the logical assumption that drugs act in their monomolecular form, then it follows that micelles, when present, will be in competition with the parasite's receptors for the monomolecular form and the final result will be as though less drug were present than had been weighed out. As an example of mixed micelle formation, the case of potassium myristate ($C_{14}$) may be considered. This substance develops micelles at one-quarter the concentration at which potassium laurate ($C_{12}$) does. As little as 15 per cent of potassium myristate halves the critical micelle concentration of potassium laurate (Klevens, 1948).

Hence it is suggested that when two interfering substances resemble one another chemically, they may be interfering

* A micelle is a type of polymer forming in concentrated solutions and reverting to monomer on dilution. The concentration at which the change-over occurs is called the critical micelle concentration. In a homologous series, the critical micelle concentration falls as the molecular weight rises.

hrough the formation of mixed micelles. As triphenyl-
nethanes have lower critical micelle concentrations than
cridines, this explanation may well apply to the case of
XXIII) interfering with (XXII). This hypothesis opens
p an entirely new field in the interpretation of interference
henomenon because it implies that interaction between
he two interfering substances occurs even in the absence
f the parasite.

Quite a different type of interference phenomenon was
liscovered by v. Jancsó and v. Jancsó (1936). These
vorkers found that in a set of twenty-two oxidation-
eduction indicators, all the members with potentials
etween +0·12 V and −0·06 V can interfere with the
herapeutic action of arsenicals and antimonials on try-
anosome-infected rats. This effect reaches its maximum
t +0·01 V, and methylene blue shows it very strongly.
These authors' explanation was that the interfering sub-
tance carried out the hydrogen-transferring work of the
nzyme which the arsenical was inhibiting. This is highly
ikely, because methylene blue has long been used by
xperimental biologists as a substitute for respiratory
nzymes which have been poisoned by cyanides. It should
e noted that ascorbic acid ($E_0 = +0{\cdot}07$) similarly inter-
eres with the action of arsenicals. Truly, the dangers of
herapeutic interference lurk in every bottle of medicine,
nd it may be that many failures in therapy could be traced
o this source.

v) *Adsorption*

In discussing therapeutic interference, the word 'adsorp-
ion' was used. Examples of adsorption have been dis-
ussed in all the chapters, although the name has been
argely avoided hitherto. Adsorption is one of those
vords that many people use without having very clear
deas about what it means. However, now that we have
o many examples on hand, a little more time might be
evoted to discussing this subject.

Adsorption was not a well-understood phenomenon until Langmuir clarified the subject in two excellent papers (1916, 1917). We now realize that adsorption is a chemical reaction taking place at a surface. The reaction involves exactly the same types of bond as are concerned with chemical reaction in the bulk phase, whether covalent, ionic, hydrogen or van der Waals' bonds. It may be asked, then, why a special term is necessary to describe this ordinary phenomenon.

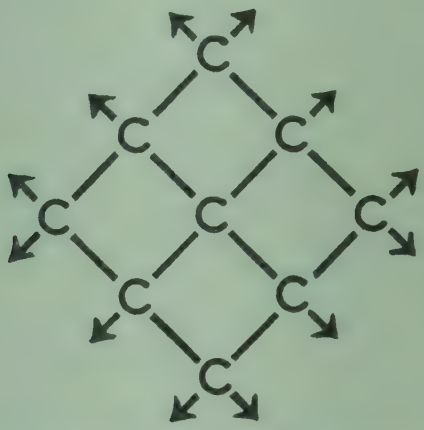


Fig. 3—Residual affinity of fragment of carbon.

A surface has two special features which can make reactions taking place there quantitatively different from analogous reactions taking place in solution. Firstly, a surface presents a 100 per cent concentration of the substance involved. As the substance is sparingly soluble (if it were not sparingly soluble, it would not be present as a surface), this concentration enormously increases the opportunities for the reaction taking place. For example, a crystal of silver chloride has a surface concentration of 7-normal, as anyone can verify from the molecular weight. On the other hand, a saturated solution of this substance contains practically no silver chloride at all.

The other special feature about a surface is that it is apt to contain unsatisfied valencies, which, elsewhere in the solid, are used to bind similar atoms or molecules together. This is evident from the representation of a piece of carbon

in Fig. 3. It is obvious that the finer the carbon is ground, the more residual valencies there will be, and the more active an adsorbent it will become.

Adsorption is usually a reversible process and equilibrium is established according to the mass action law. Langmuir derived an equation from this law to permit of a more accurate quantitative treatment of adsorption than had been possible hitherto. This 'isotherm' as it is called, is represented graphically as a hyperbola and denotes eventual saturation of the appropriate receptor groups on the surface.

All who are interested in selective toxicity are constantly brought up against adsorption phenomena. Even in an apparent solution like human serum, there are 100 square metres of protein surface in every cubic centimetre!

*CHAPTER EIGHT*

# SELECTIVE TOXICITY IN RELATION TO PHARMACOLOGY

## (i) *Pharmacology and chemotherapy compared*

Pharmacology is unique among the branches of selective toxicity in that both the economic and the uneconomic species are constituent cells of the one organism. Hence, difficult problems arise which have little or no counterpart in, say, chemotherapy.

In the first place, pharmacological results are usually required to be reversible. The patient who submits to an anaesthetic does not expect to be deprived of feeling for the rest of his life. In chemotherapy, on the other hand, the toxic action is most esteemed when it is most irreversible. Only in the eradication of cancer does the pharmacologist seek to incapacitate the uneconomic tissue permanently.

In the second place, pharmacological drugs are expected to supply a graded response. In proportion to the severity of a spasm or excessive secretion, so should various doses of the remedy exactly neutralize what is morbid without inflicting upon the patient a total loss of function. The reverse of the graded response, viz. the all-or-nothing effect, is undesirable in pharmacology but unobjectionable in chemotherapy.

In the third place, the pharmacologist, compared with the chemotherapist, has greater difficulty with his biological testing material. Usually he finds it harder to isolate in quantity and in a uniform condition. In exploring any branch of selective toxicity it is ideal to begin with the simplest system possible, viz. the selectively toxic agent*

* Moreover, care should be exercised that only one species of this is present: see Chapter IV.

nd a uniform population of the cells on which it is to act. Once these fundamental relationships have been painsakingly explored, the natural complicating factors may be dded gradually so as to enable the work eventually to be pplied to some utilitarian end. Thus one may progress rom a uniform population of effector cells to the tissue n which they occur, from these to the organ and eventually o the entire plant or animal.

However, the pharmacologist finds difficulty in studying is effector cells in isolation. For example, he may be tudying the effect of curare on those few molecules which ccur at one end of a muscle-cell just where the end of a erve-cell comes within a minute distance of them. Naturally, it is not practicable to isolate these receptor-nolecules, or even the whole neuromuscular junction; ut the pharmacologist actually has not found it convenient o work with anything less than what he calls a 'nerve-nuscle preparation', which contains many nerve-cells and nuscle-cells in a far from uniform condition. However, s pharmacologists' test-objects go, this nerve-muscle reparation is remarkably free from foreign types of cells. A great deal of exploratory pharmacological work is done n perfused organs and even on the intact animal.

In passing, mention should be made of one branch of harmacology which can hardly be classed as selective oxicity. This is replacement therapy, in which medicinal se is made of a normal bodily constituent which is emporarily or permanently absent, e.g. a vitamin, a ormone or an inorganic ion.

One often hears it said that a good chemotherapeutic gent should have no pharmacology, meaning that it should ave no unpleasant side-effects on the host, and penicillin quoted as the only known example of this ideal state. owever, this definition is faulty: many pharmacological roperties can be helpful, such as those which send the rug to the site of action. If penicillin could get into the d cells of the blood, it might well cure malaria.

(ii) *The value of measurement*

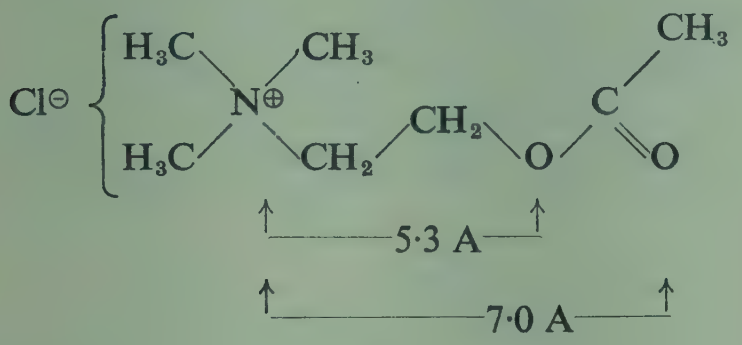
The late A. J. Clark, perhaps more than any other person, brought to pharmacology an appreciation of the help that the physical sciences could give. Clark taught the importance of exact measurement in pharmacology, the need for standardization of technique and the use of statistics in interpreting results. Above all, he urged workers to visualize the action of drugs in terms of the relative shapes and sizes of molecules and cells.

The importance of these size relationships in chemotherapy has already been mentioned and a suitable mnemonic diagram forms the frontispiece to this book. It is expected that the reader will now have memorized these dimensions and is ready to learn a few more. A molecule of protein, if fully unfolded, would be about 8 mμ long and a muscle-cell about 60 μ in length. Let us look at these figures in another way. If we make a Hirschfelder model of some common pharmacological drug (the scale of these models is 0·1 mμ to 1 cm.), the molecule of the drug will seem to be about 2 inches long, a molecule of muscle protein 10 yards long, and a muscle-cell 3½ miles long by 200 yards wide (cf. Clark, 1933). These size relationships give us some idea of the small probability of a drug hitting the desired receptor. Yet the required adsorptions do take place, in some cases even at a dilution of $10^{-9}$ M, a dilution at which very few substances can be precipitated in an ordinary chemical test.

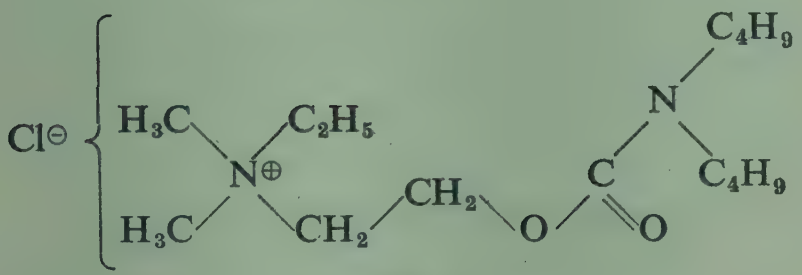
Recently, some interesting attempts have been made to correlate molecular dimensions and pharmacological activity. Schueler (1946) has stated that all oestrogenic substances have two hydrogen-bonding groups, 8·55 Å apart, and that this is responsible for the nature of their activity. Actually the bondable hydrogens of the two hydroxyl-groups in stilboestrol are 15·5 A apart so that it is not easy to see what this author had in mind, but this dimensional type of thinking is to be encouraged

particularly if it can be tied up with known interatomic distances in proteins and nucleic acids.

Pfeiffer (1948) pointed out that certain drugs which stimulate the parasympathetic nerve-endings adjacent to involuntary muscle (i.e. muscarinic parasympathomimetics) had quite characteristic dimensions as shown in (I).




(I)
Acetylcholine




(II)
Dibutoline

Not only acetylcholine, but many substances like muscarine, doryl and mecholyl, which mimic its action on the non-peripheral parasympathetic nerve-endings, contain a basic nitrogen atom with a ketonic oxygen adjacent to an ether oxygen at a distance of two saturated carbons (*c.* 5·3 A). This proves, however, to be a suggestive rather than an inclusive definition, because butyrylcholine is inactive (Ing, 1949) and alkyl trimethylammonium salts have true parasympathomimetic activity, even if of comparatively small degree (Burn and Dale, 1914).

Pfeiffer also pointed out that substances, e.g. dibutoline (II) or atropine (III), which antagonize this muscarinic action, can also be represented by formula (I), except that the acyl-group now carries a large instead of a small substituent. The usual relationship between metabolite and metabolite-analogue is revealed here, and the ensuing antagonism is not surprising. It has been shown that the replacement of even a single methyl by ethyl in acetylcholine, decreases its activity, and that, when the three methyl-groups have been replaced in this way, the activity is changed not only in degree but also in nature (Holton and Ing, 1949).

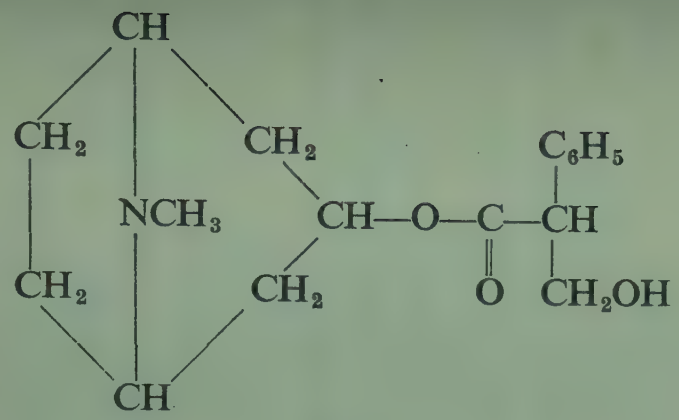
(iii) *Simplification of structure*

It is always essential, in tackling a problem in selective toxicity, to find out what is the simplest structure that has the typical activity under investigation. Many naturally occurring alkaloids have powerful pharmacological actions and much success has attended attempts to obtain simpler analogues (cf. Goodman and Gilman, 1941). The simpler substances are easier to manufacture in quantity, and are usually free from undesirable side-effects which the more complex naturally-occurring substances possess.

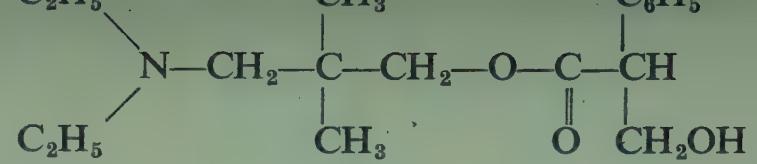
In this connexion, it is interesting to observe how far the molecule of atropine (III) has been unpicked until just the right degree of complexity remains as in 'syntropan' (IV) and 'trasentin' (V), which are good antimuscarinic drugs, like atropine. The guiding principle here, as elsewhere, is that the nitrogen in a *saturated* heterocyclic ring is well ionized at pH 7, and hence can be replaced by an aliphatic amino-group.

Cocaine (VI) was similarly simplified to procaine (VII), which is only one-tenth as active, and this has been modified to give 'butyn' (VIII), which is quite as potent a local anaesthetic as cocaine.

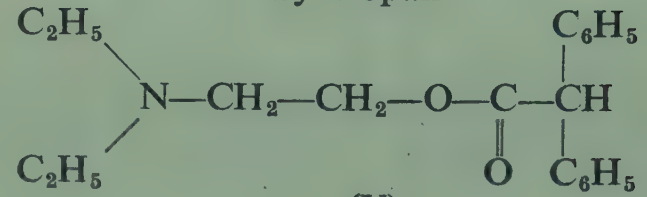
The simplification of the molecule of curarine (IX), which is used to relax voluntary muscles, remained an



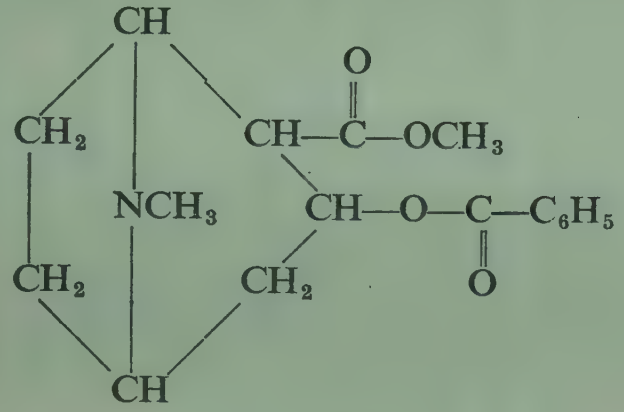
(III)
Atropine



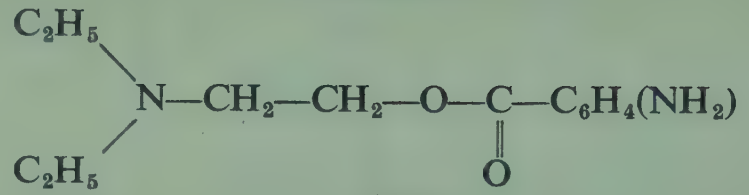
(IV)
'Syntropan'



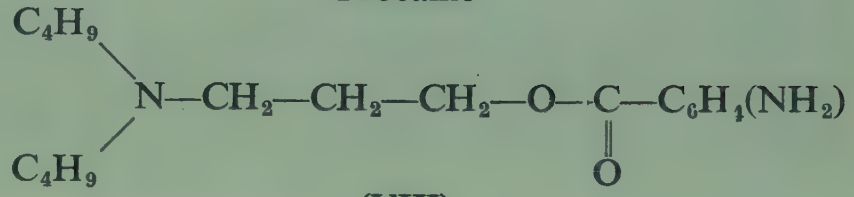
(V)
'Trasentin'



(VI)
Cocaine



(VII)
Procaine



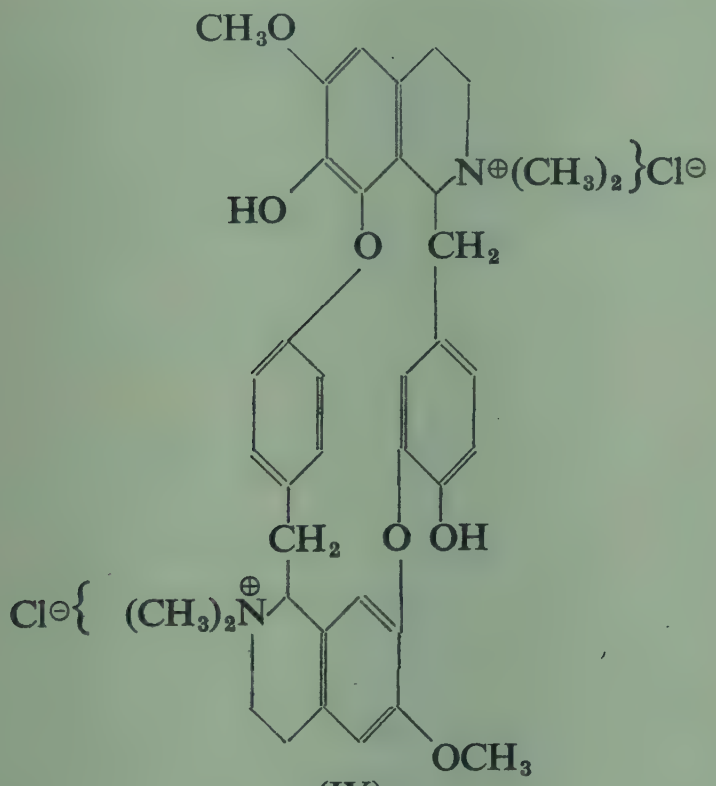
(VIII)
'Butyn'

unsolved problem until quite recently. This delay wa partly due to the fact that the detailed structure of curarin has not long been known (King, 1948), but it was als due to the very complexity of the molecule. Just as it too patient work to discover that the methyl ester group i cocaine was redundant, so, in seeking essentials in th much more intricate structure of curarine, many blin alleys were followed. In turn the action was sought i compounds with a quaternary nitrogen atom, in tetra hydroquinolines, in benzyl-derivatives and more recentl in amino-ethers. Mono-quaternary ammonium compound do not possess the effect in a sufficiently high degre to be useful (Ing, 1936). Nevertheless, simple aliphati *bis*quaternary compounds, particularly decane*bis*trimethy ammonium iodide (X) have been found to have pote curare-like properties (Paton and Zaimis, 1948; Barlow an Ing, 1948) and have found instant clinical application. Th di-esters of choline with dibasic acids, even with succin acid, also give powerful curariform drugs of clinical valu (Bovet and Bovet-Nitti, 1949).

As was pointed out by Ing (1936), curarine acts b occupying acetylcholine-receptors on the voluntary musc at neuromuscular junctions, thus preventing acetylcholin from transmitting the nervous impulse (acetylcholine secreted by the adjacent nerve-ending). Curiously enough acetylcholine is itself curarizing in abnormally large dose This fact suggests that it is effective only when it can mak a two-point contact with the receptors; when present in to high a concentration, so many molecules of acetylcholin are making one-point contacts that there is steric hindranc to the establishment of two-point contacts.

Thimann (1943) pointed out that there was a good de of evidence that procaine (VII), and similar local anae thetics act by blocking receptors used by acetylcholin at sensory nerve-endings and, possibly, in mediatin the nervous impulse. The antagonism between acety choline and the atropine series of drugs at the junction

parasympathetic nerves and involuntary muscle has already been mentioned. Here, then, we find three distinct classes of drugs, each having its chemical individuality, and each evoking a distinct physiological response. Yet in each case the evidence points to the ultimate action being identical in so far as acetylcholine is prevented from discharging its normal function. This suggests very strongly that the chemical individuality of each of these classes of drugs is concerned with distributing the drug to the correct site of action. In short, it must confer the correct adsorption and partition properties, both during transit to the site and at the site itself. This line of thought has great significance for the future development of pharmacology and offers a valuable point of attack for the surface-chemist.



(IX)
Curarine

$$I^{\ominus}\{(CH_3)_3N^{\oplus}(CH_2)_{10}N^{\oplus}(CH_3)_3\}I^{\ominus}$$

(X)
Decamethonium iodide

Burn (1948) has extended this list by suggesting that pethidine and other analgesics act by antagonizing acetylcholine in the central nervous system and that quinidine lengthens the refractory period of the heart by antagonizing the important contribution of acetylcholine to the metabolism of heart muscle. In the following list of chemically and pharmacologically distinct substances, every member has all of the following properties (although each member has one of these properties highly developed at the expense of the others). *Substances*, atropine, procaine, quinidine, pethidine, papaverine and sparteine; *properties*, local anaesthetic, spasmolytic, analgesic, prolongation of refractory period of heart and general antagonism of the action of (experimentally added) acetylcholine on a number of different tissues.

Drugs are also known which have the opposite action, namely they reinforce the action of naturally secreted acetylcholine. Physostigmine (XI) is an alkaloid which, even at a dilution of $10^{-7}$ molar, powerfully inhibits the enzyme choline-esterase whose normal function is to destroy acetylcholine, thus confining the action of this important transmitter of the nervous impulse to the instant of liberation. Hence, physostigmine has the effect of giving the patient an overdose of his own acetylcholine (Loewi and Navratil, 1926). This action is particularly evident at the neuromuscular junction and hence physostigmine is a direct antagonist of curarine. Stedman (1926) was able to show that the characteristic action of physostigmine was shown by the methylurethane of phenol; thus only a small part of the molecule of the alkaloid is essential. He showed that the effect is intensified by a basic group which need not necessarily be placed *para* to the phenolic group (as in physostigmine). As an amino-group, attached directly to an aromatic ring, can be ionized at pH 7 only if it is first made quaternary, it is evident that 'prostigmine' (XII) represents the greatest simplification of physostigmine which can be expected. Actually, 'prostigmine', being free

om the undesirable chemical and pharmacological pro-
rties of the natural alkaloid, is widely used in place of
ysostigmine.

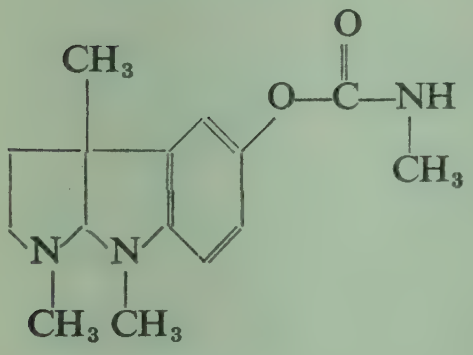
The main pharmacological effects of strychnine and some
-effects of morphine (emetic action, central stimulation)
ve been ascribed to the known inhibitory action of these
bstances on choline-esterase (cf. Bernheim, 1942). Here
ain, as with the acetylcholine receptor-blocking series of
ugs described above, we obtain a hint of how differential
tribution produces varied pharmacological effects, even
ough these may depend on a chemically identical
action.

Following on Loewi's demonstration of the mode of
tion of physostigmine, Gaddum and Kwiatkowski (1938)
ggested that some of the pharmacology of ephedrine was
e to its known ability to inhibit amine-oxidase, an
zyme which destroys adrenaline: thus the patient received
overdose of his own adrenaline. Some difficulties in the
y of accepting this hypothesis have been summarized by
lquist (1949), but these difficulties largely disappear if
original hypothesis is expanded to include *nor*adrena-
e as well as adrenaline (J. H. Gaddum, private com-
unication).

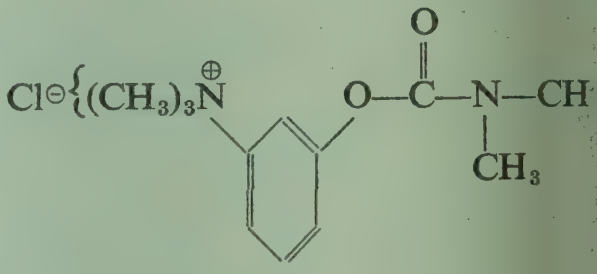
Morphine (XIII) is a complex alkaloid for which a
mpler analogue was vainly sought until Eisleb and
naumann produced pethidine (XIV) in 1938. This was
lowed, within a few years, by the discovery of a non-
terocyclic analogue, amidone (XV), which is even more
werfully analgesic than morphine. The history of this
rk, and the connexion between constitution and action
ong these analgesics, has been recently reviewed (Bergel
d Morrison, 1948). It is now realized that in the mole-
e of morphine (XIII), one pair of rings is almost at
nt angles to the others. It has been suggested that
structural features common to morphine, pethidine,
done and their active analogues are (i) a basic
iary nitrogen atom, (ii) a water-attracting group and

(iii) a central carbon atom so substituted that free rotation of the component groups is inhibited, resulting in a grossly three-dimensional structure (Anker and Cook, 1946).

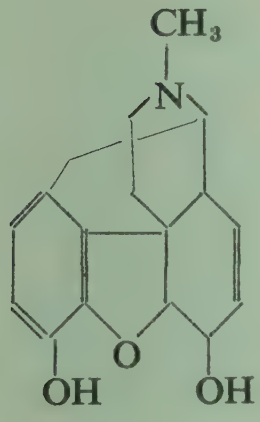
At the present time, a serious attempt is being made to simplify the ergot alkaloids. Clinically the most important of these is ergometrine (XVI). The typical contracting action of ergometrine on the uterus has been obtained with a simple ester (XVII), although the action is 20 times weaker (Baltzly, Dvorkovitz and Phillips, 1949).



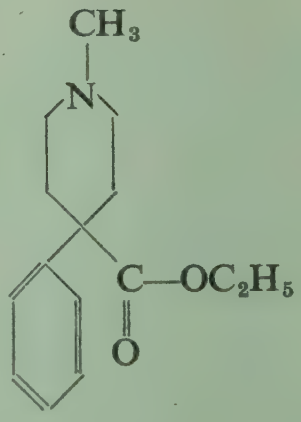
(XI)
Physostigmine



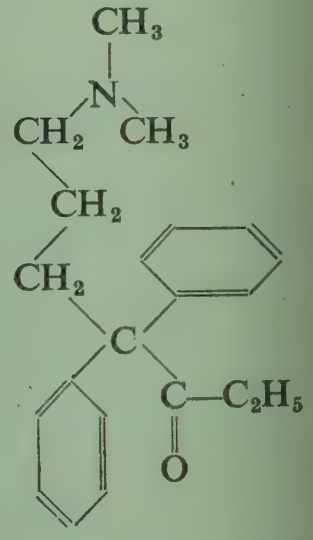
(XII)
'Prostigmine'



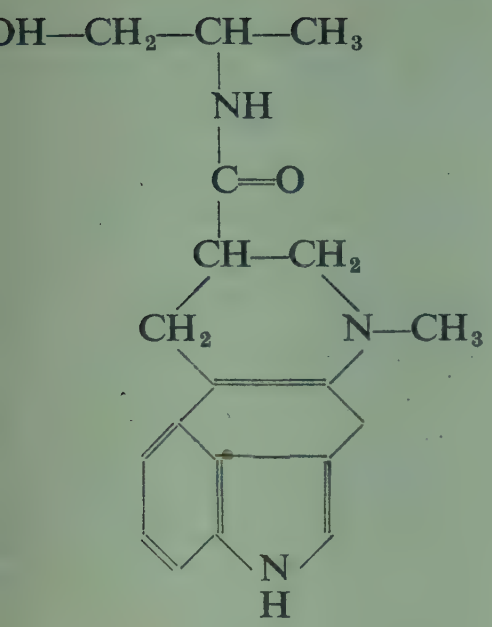
(XIII)
Morphine



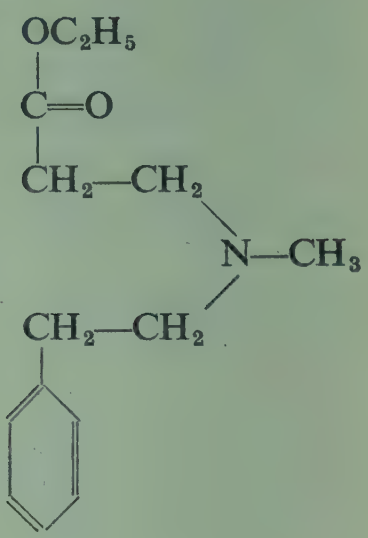
(XIV)
Pethidine



(XV)
Amidone



(XVI)
Ergometrine



(XVII)
2-Phenyl-2′-carboethoxy-diethylmethylamine

(iv) *Structurally non-specific drugs*

In the preceding section we have seen that drugs which can compete with acetylcholine can apparently have a multiplicity of pharmacological actions, depending upon the tissues in which their partition coefficients cause them to become concentrated. The majority of these substances, like acetylcholine itself, are derivatives of 2-aminoethanol, and hence structural considerations are of great importance in this series.

On the other hand, the depressants of the central nervous system (i.e. the hypnotics and general anaesthetics) have, for a long time, mystified investigators by the apparent lack of connexion between structure and action. Satisfactory drugs with these actions are found among the alcohols, ketones, sulphones, acid amides (including cyclic acid amides such as the barbiturates), ethers, chlorinated hydrocarbons and even the hydrocarbons themselves.

Overton (1901) and Meyer (1899) independently concluded that depressant action resulted when a substance

had a moderately high oil/water partition coefficient. This means that a potent hypnotic, when shaken with a mixture of olive oil and water, should accumulate in the oil to an extent from 1 to 100 times as great as in the water. In the limited series examined by these workers, it was found that the more powerful the hypnotic, the higher was the partition coefficient. The principal exception was chloral hydrate, whose coefficient was too low, but this anomaly is no longer puzzling because it has recently been shown that chloral hydrate does not act until it is converted to trichloroethanol which has an acceptably high coefficient (Butler, 1948). Overton and Meyer believed that the high lipoid content of nerve tissue caused depressants to accumulate in it, because of their 'lipophilic' properties.*

In spite of these apparent concordances, the Overton-Meyer relationship is now known not to be widely valid. A partition coefficient of 1 between olive oil and water is given by phenobarbitone (a very strong hypnotic), by butanol (a very weak hypnotic) and by 5-aminoacridine (which has analeptic, i.e. anti-hypnotic, properties). The use of olive oil, moreover, is questionable as it is entirely unlike the lipoid constituents of cell-membranes, e.g. cholesterol and cephaelin. Finally, substances with very high partition coefficients (e.g. olive oil itself) are not at all depressant.

A new correlation between depressant action and a physical property was put forward by Traube in 1904. Traube found that the greater the depressant action, the greater the lowering of surface-tension at an air/water interface. The converse, however, was found untrue, and Traube's relationship usually applies only to the members of an homologous series. Little store can be set upon this correlation for, as was pointed out in Chapter IV, a low surface-tension at the air/water interface is merely a measure of the non-wettability of the substance and does

* Aeschlimann (1936) has pointed out that substances with partition coefficients that are somewhat too low to confer depressant properties, often have strong analeptic (i.e. stimulant) properties, e.g. tetra-ethylurea.

ot imply any specific adsorbability on a cellular receptor. Moreover, as Davson and Danielli (1943, p. 64) show, the urface-tension between a cell-membrane and the water by hich it is surrounded is zero, so that no further lowering conceivable.

The cellular membrance is now recognized as playing an ssential role in the propagation of excitation: hence it is likely site for the action of hypnotics. It is known that ypnotics do not act as depressants when injected into ells, but only when allowed to act from without. Hence Varburg (1921) suggested that depressant drugs act by oating the outside of cells, thus inhibiting oxidative pro- esses occurring there very much as a coating of grease revents a piece of iron from rusting.

Quastel (1939) showed that only certain oxidative pro- esses were inhibited by depressants. In the brain, the xidation of pyruvate, glucose and lactate were affected, hereas that of succinate was not. He believed that epressants act by disorganizing the space relationships of onsecutive enzymes in an oxidative chain, particularly etween a riboflavine-containing enzyme and the cyto- hrome component immediately responsible for its ehydrogenation.

The toxic action of structurally non-specific substances as discussed in general terms in Chapter II, and it was hown that Ferguson's Principle of fractional saturation atisfactorily accounted for the biological effect obtained. On the lower animals, this effect is a depressant one followed by death if the dose is increased). Many a ubstance whose action on small animals is known to be overned by Ferguson's Principle is a hypnotic or general naesthetic for man. Recent investigations of the action f depressants on the nervous system of mammals have isclosed that the degree of hypnotic and anaesthetic ction which they show is governed by Ferguson's rinciple (Brink and Posternak, 1948).

The rest of the story appears to involve an accumulation

of the drug in the membrane resulting in a disorganization of enzyme sequences, as postulated by Quastel. (For a review of current knowledge of the mode of action of general anaesthetics, see Butler, 1950).

(v) *Selective toxicity and tumours*

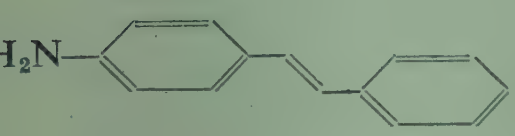
Specialists in cancer research consider that suppression of cancerous growths without injury to the host is the most difficult of all the branches of selective toxicity. However, hope of success is held out by the chemical differences between cancerous and normal tissues, such as the large excess of thiamine in leukaemic leucocytes.

Certain successes have already been obtained in this field. For example, stilboestrol is regularly and successfully used to suppress cancer of the prostate. This action is due to gynergen/androgen competition: the prostatic epithelium, whether healthy or malignant, vanishes when no effective supply of androgen is available. The reciprocal use of androgens in female cancer of the sex organs has not been very successful but it must be recalled that known androgens are neither potent nor cheap.

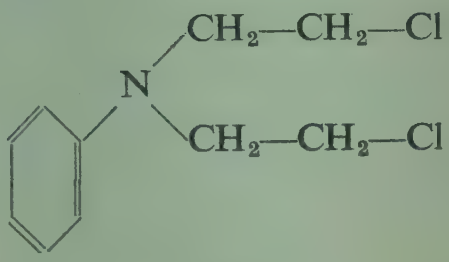
In leukaemia, which is broadly speaking a cancer of the white blood cells, urethane has been given with some success since its first use for this purpose by the Royal Cancer Hospital, London, in 1943 (cf. Haddow and Sexton, 1946). Life is definitely prolonged, but the remission lasts only as long as the treatment. It has been suggested that urethane competes with a component used by the malignant cell to synthesize pyrimidine rings for the nucleic acid which is present so abundantly (Todd, 1946).

In Hodgkin's disease, which is characterized by malignant lymphatics, the nitrogen-mustards have been found to alleviate the condition. The most effective members of this series are those, such as (XIX), which have an aromatic nucleus attached to the nitrogen (Haddow, Kon and Ross, 1948). The action is intensified by the presence of *two* fairly easily hydrolysed chlorine atoms. The distance

etween the chlorine atoms is somewhat critical and it is uggested that molecules of this type act by forming nbiological cross-linkages in chromosomes (Goldacre, oveless and Ross, 1949). An alternative explanation is hat the nitrogen-mustards (and the biologically similar oly-epoxides and poly-ethyleneimines) polymerize to yield inear structures with reactive alkylating groups spaced at egular intervals. It is thought that these polymers may ct as unbiological analogues of nucleic acid, thus causing he gross mitotic irregularities which are always seen ollowing the use of these substances (Rose, Hendry and Walpole, 1950).



(XVIII)



(XIX)

Derivatives of 4-aminostilbene (XVIII) have given esults in leukaemia and Hodgkin's disease only slightly nferior to those shown by the aromatic nitrogen-mustards. 4-Dimethylaminostilbene is one of the most effective nembers of this series. A second water-attracting group n the other ring decreases the action as does hydrogenation of the central double bond or the presence of any *ortho*-substituent which prevents the rings from lying flat (Haddow, Harris, Kon and Roe, 1948).

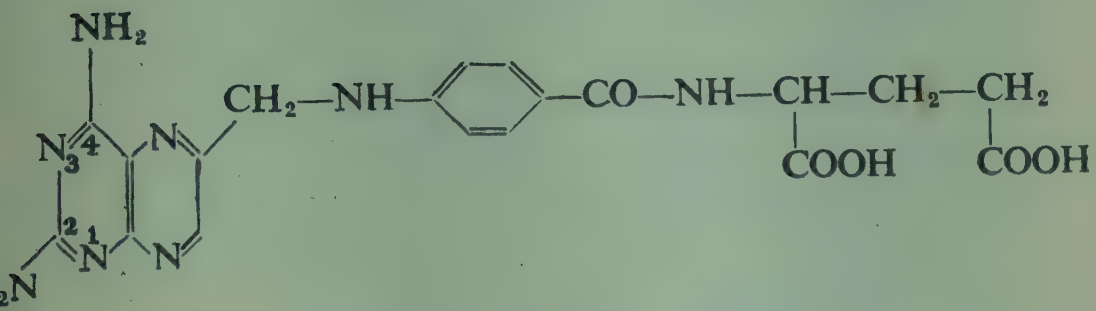
Because pteridines are believed to play an important part n the synthesis of nucleic acid, various analogues of folic acid have been tested as tumour-inhibiting agents. The most successful of these is aminopterin (XX) which differs from folic acid only in having a $-NH_2$ group in the 4-position where folic acid has an $-OH$ group (Franklin, Stokstad and Jukes, 1948). It has been suggested, in

Chapter V, that a hydroxy-group in position 4 may be essential for the normal working of the natural pteridines, because of its metal-carrying properties. Hence, it is not surprising to learn that aminopterin is a powerful antagonist of folic acid in a number of organisms and tissues, including cancer tissue. Unfortunately the margin between the active and the toxic dose is so small that it has been little used.

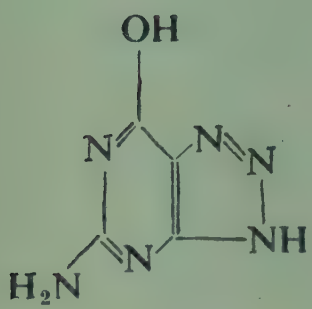
Much work has been done with various purine analogues made by G. H. Hitchings. Of these, the most promising is 8-azaguanine (XXI), known in the United States as guanazolo. Mammals ordinarily synthesize the guanine component of nucleic acid but cannot utilize guanine as such. Some more primitive organisms, however, cannot synthesize the guanine component and require guanine (XXII) to be supplied to them as such. Hence, analogues of guanine, e.g. guanazolo, are lethal to these organisms. Malignant tissues appear also to have a metabolism of this more primitive type and to be strongly inhibited by guanazolo (Kidder, Dewey, Parks and Woodside, 1949). This substance differs from aminopterin in being relatively non-toxic to mice.

The relation between structure and action in tumour-producing substances is better known than in the tumour-arresting substances. The effect of methyl-groups in conferring cancer-producing properties on polycyclic hydrocarbons has been extensively studied. It has been pointed out that the presence of a phenanthrene bridge (e.g. the 3:4-bond in 1:2-benzanthracene, (XXIII) is a common attribute of such substances and is apparently essential for their activity (Robinson, 1946). Benzanthracene, itself, is not carcinogenic, but it becomes so when a methyl-group is inserted in the 5-, 6-, 9- or 10-position (Barry *et al.*, 1935). Of these, the 9- and 10-positions have by far the strongest effect. If, however, the phenanthrene bridge is substituted (e.g. by placing a methyl-group in the 3-position), carcinogenic properties are reduced to a low

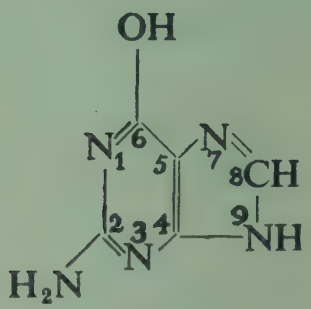
level. This is understandable if the phenanthrene bridge must be kept free of atoms which, by their bulk, would impede union with some cellular constituent.



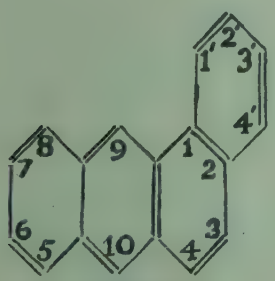
(XX)
Aminopterin



(XXI)
Guanazolo



(XXII)
Guanine



(XXIII)

It has been claimed that carcinogenic properties do not appear in polycyclic hydrocarbons until the electron-density of the phenanthrene bridge* has been brought up

* Also known as the K region.

to 1·28 *e* by appropriate substitution (e.g. by a suitably placed methyl-group): in proportion as the electron-density rises so does carcinogenic activity increase (Pullman, 1945, 1946, 1947). Badger (1949) has criticized this hypothesis as applied to benzanthracenes and benzphenanthrenes on two grounds: (i) the 1′-, 2′-, 3′- and 4′-methyl-1 :2-benzanthracenes are inactive, although they have a sufficiently charged phenanthrene bridge; (ii) Pullman's biological grading is unjustifiably high for many substances, which, when regraded by current British standards, often present serious objections to the hypothesis.

It has been claimed that the cancer-producing properties of various polycyclic hydrocarbons can be inhibited by the application of (relatively) harmless chemical analogues (Lacassagne, Buu-Hoï and Rudali, 1945). However, no therapeutic applications of this discovery have been found.

# EPILOGUE

IN concluding this brief account of selective toxicity, it is logical to pause and ask what arrangements are being made for the future growth of the subject.

It might be thought that firms who are manufacturing selectively toxic agents would be, singly or collectively, making a large-scale systematic search for fundamental principles. However, such is not the case. In Great Britain and in the United States the individual firms tend to direct their research activities along lines which are likely to produce marketable products at an early date. This policy is urged on by the spur of competition not only from other firms within each country, but also from international rivals. Moreover, in the trade there is a strong, if ill-founded, feeling that sufficiently rapid progress can be made along purely empirical lines: if fundamental studies are worth pursuing, the universities should undertake them. This was, of course, the attitude of industry in pre-war Germany in spite of the almost complete freedom from internal competition. The many remarkable discoveries of 'Bayer' (and other component firms of I. G. Farben-industrie) were made principally by the lavish expenditure of money and man-power on ventures of trial and error. Moreover, by a system of grants and rewards, many able brains in the universities were induced to divert their talents from serious fundamental studies to short-term projects. However, it is a fact that pieces of good fundamental research in selective toxicity have occasionally been carried out in the chemical industry. Notable examples are Ferguson's work in England on the mode of action of structurally non-specific chemicals, Veldstra's work in Holland on synergism, and Bell and Roblin's work in the U.S.A. on the connexion between ionization and action in sulphonamides. However, industry is not yet, to any

appreciable extent, furnishing a congenial climate for this type of work.

Hence, for the next few years at least, fundamental scientific work on selective toxicity will be carried out almost entirely in universities and national research institutions, sometimes with the help of grants from the more far-sighted sections of the industry, yet sometimes in the teeth of specific inducements from industry to concentrate on *ad hoc* projects contrary to their long-term interests.

It cannot be assumed that, in the immediate future, universities and national research institutions will launch large-scale programmes of research into the principles of selective toxicity. As far as universities are concerned, selective toxicity does not rank as a primary category of learning. True, the disciplines which its study requires are likely to be available separately in the various departments of a university but the possibility of combining them into a unified course will not often present itself to the mind of a likely student. Research institutions usually relate their research programmes to specific topics, such as the chemotherapy of tuberculosis or the destruction of the flour-moth. No research institution exists anywhere in the world for the study of the principles of selective toxicity, and existing institutions are not yet devoting any considerable portion of their funds to such studies which require the organized co-operation of physical and organic chemists with biochemists and many categories of biologists.

This state of affairs need not be permanent. Those who have the interests of this subject at heart, as many readers of this book must have, will constantly be striving to co-operate in the advancement of so important a branch of science. Much can be done by the regular meeting of chemists and biologists for informal discussions designed to initiate new work in this field or to make better known the results which have already been established.

# APPENDICES

# APPENDIX I

## CONNEXION BETWEEN IONIZATION AND ANTIBACTERIAL ACTIVITY IN THE ACRIDINE SERIES. HIGHEST DILUTIONS COMPLETELY PREVENTING VISIBLE GROWTH IN 48 HOURS AT 37° C.

### Medium: Broth containing 10 per cent of serum; pH 7·2—7·4

Key to dilutions:
0 signifies growth at 1 in 5,000
1 signifies inhibition at 1 in 5,000
2 ,, ,, ,, 10,000
3 signifies inhibition at 1 in 20,000
4 ,, ,, ,, 40,000
5 ,, ,, ,, 80,000
6 ,, ,, ,, 160,000
7 signifies inhibition at 1 in 320,000
8 ,, ,, ,, 640,000
9 ,, ,, ,, 1,280,000
10 ,, ,, ,, 2,500,000

| *No.* | *Substance* | *Organisms* | | | | | *Bacteriostatic Index (sum total of code number of inhibitory dilutions)* | *$pK_a$ in water at 37°* | *Per cent ionized (kation) at 37° C. and pH 7·3* |
|---|---|---|---|---|---|---|---|---|---|
| | | Cl. welchii | Strept. pyogenes | Staph. aureus | B. coli | Proteus | | | |
| 1 | Acridine | 3 | 1 | 1 | 0 | 1 | 6 | 5·3 | 1·0 |
| 2 | 1-Methylacridine | 2 | 0 | 0 | 0 | 0 | 2 | 5·2 | 0·9 |
| 3 | 3-Methylacridine | 0 | 0 | 0 | 0 | 0 | 0 | 5·9 | 4·0 |
| 4 | 5-Methylacridine | 3 | 0 | 0 | 0 | 0 | 3 | 6·0 | 5·0 |
| 5 | 1:3:4:6:7:9-Hexamethylacridine | 0 | 0 | 0 | 0 | 0 | 0 | 4·3 | <0·1 |
| 6 | 1-Methoxyacridine | 3 | 0 | 0 | 0 | 0 | 3 | 4·6 | 0·2 |
| 7 | 3-Methoxyacridine | 3 | 2 | 1 | 0 | 0 | 6 | 4·7 | 0·3 |
| 8 | 2:8-Dimethoxyacridine | 5 | 4 | 0 | 0 | 0 | 9 | 6·1 | 6·0 |
| 9 | 1-Acetamidoacridine | 2 | 1 | 0 | 0 | 0 | 3 | 2·9 | <0·1 |
| 10 | 2-Acetamidoacridine | 2 | 2 | 0 | 0 | 0 | 4 | 5·5 | 1·6 |
| 11 | 3-Acetamidoacridine | 4 | 2 | 1 | 1 | 1 | 9 | 4·6 | 0·2 |
| 12 | 4-Acetamidoacridine | 0 | 0 | 0 | 0 | 0 | 0 | 4·3 | 0·1 |
| 13 | 5-Acetamidoacridine | 0 | 0 | 0 | 0 | 0 | 0 | 4·2 | <0·1 |
| 14 | 1-Hydroxyacridine | 5 | 4 | 3 | 0 | 0 | 12 | 4·6 | 0·2 |
| 15 | 2-Hydroxyacridine | 3 | 2 | 1 | 0 | 0 | 6 | 5·3 | 1·0 |
| 16 | 3-Hydroxyacridine | 0 | 0 | 0 | 0 | 0 | 0 | 4·6 | 0·2 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 18 | 9-Hydroxyacridine (acridone) | 0 | 0 | 0 | 0 | 0 | 0 | <2 | <0·1 |
| 19 | 1-Aminoacridine . . | 2 | 1 | 1 | 0 | 0 | 4 | 4·2 | <1 |
| 20 | 2-Aminoacridine . . | 6 | 5 | 3 | 4 | 3 | 21 | 7·7 | 73 |
| 21 | 3-Aminoacridine . . | 5 | 2 | 1 | 0 | 0 | 8 | 5·6 | 2 |
| 22 | 4-Aminoacridine . . | 5 | 2 | 1 | 1 | 0 | 9 | 5·7 | 2 |
| 23 | 5-Aminoacridine (Acramine, B.P.) . . | 7 | 6 | 4 | 4 | 4 | 25 | 9·6 | 100 |
| 24 | 3-Aminoacridan . . | 2 | 2 | 1 | 0 | 0 | 5 | 5·1 | 0·6 |
| 25 | 1:5-Diaminoacridine . . | 5 | 5 | 4 | 4 | 4 | 22 | 9·0 | 98 |
| 26 | 1:9-Diaminoacridine . . | 0 | 0 | 0 | 0 | 0 | 0 | 3·8 | <1 |
| 27 | 2:5-Diaminoacridine . . | 7 | 6 | 2 | 2 | 0 | 17 | 11·1 | 100 |
| 28 | 2:6-Diaminoacridine . . | 7 | 7 | 4 | 4 | 4 | 26 | 9·0 | 98 |
| 29 | 2:7-Diaminoacridine . . | 7 | 6 | 4 | 5 | 4 | 26 | 7·8 | 76 |
| 30 | 2:8-Diaminoacridine (Proflavine, B.P.) . . | 7 | 6 | 4 | 3 | 2 | 22 | 9·3 | 99 |
| 31 | 2:9-Diaminoacridine . . | 4 | 3 | 0 | 2 | 0 | 9 | 7·0 | 33 |
| 32 | 3:5-Diaminoacridine . . | 7 | 6 | 2 | 2 | 1 | 18 | 10·0 | 100 |
| 33 | 3:7-Diaminoacridine . . | 5 | 3 | 1 | 0 | 0 | 9 | 5·8 | 3 |
| 34 | 4:5-Diaminoacridine . . | 5 | 5 | 3 | 3 | 2 | 18 | 10·7 | 100 |
| 35 | 3-Amino-5-methylacridine . | 5 | 3 | 2 | 0 | 0 | 10 | 5·8 | 3 |
| 36 | 5-Amino-1-methylacridine . | 7 | 7 | 5 | 4 | 4 | 27 | 9·8 | 100 |
| 37 | 5-Amino-2-methylacridine . | 6 | 6 | 4 | 3 | 2 | 21 | 9·8 | 100 |
| 38 | 5-Amino-3-methylacridine . | 7 | 6 | 4 | 4 | 2 | 23 | 9·6 | 100 |
| 39 | 5-Amino-4-methylacridine . | 6 | 7 | 4 | 4 | 4 | 25 | 9·6 | 100 |
| 40 | 5-Amino-1:3-dimethyl-acridine . . . | 8 | 7 | 5 | 3 | 1 | 24 | 10·3 | 100 |

APPENDIX I—*contd.*

| *No.* | *Substance* | *Organisms* | | | | | *Bacteriostatic Index (sum total of code numbers of inhibitory dilutions)* | *$pK_a$ in water at 37°* | *Per cent ionized (kation) at 37° C. and pH 7·3* |
|---|---|---|---|---|---|---|---|---|---|
| | | Cl. welchii | Strept. pyogenes | Staph. aureus | B. coli | Proteus | | | |
| 41 | 5-Amino-1:9-dimethyl-acridine | 8 | 7 | 5 | 4 | 3 | 27 | 9·1 | 98 |
| 42 | 5-Amino-1-ethylacridine | 7 | 6 | 4 | 3 | 2 | 22 | 10·0 | 100 |
| 43 | 2:8-Diamino-1:9-dimethyl-acridine | 9 | 8 | 6 | 4 | 3 | 30 | 8·8 | 97 |
| 44 | 2:8-Diamino-3:7-dimethyl-acridine | 8 | 6 | 4 | 3 | 0 | 21 | 9·8 | 100 |
| 45 | 2:8-Diamino-4:6-dimethyl-acridine | 8 | 8 | 2 | 4 | 0 | 22 | 10·2 | 100 |
| 46 | 2-Amino-5-chloroacridine | 1 | 0 | 0 | 0 | 0 | 1 | 6·4 | 11 |
| 47 | 2-Amino-7-chloroacridine | 5 | 4 | 3 | 1 | 0 | 13 | 6·7 | 20 |
| 48 | 2-Amino-8-chloroacridine | 4 | 4 | 3 | 2 | 0 | 13 | 7·0 | 33 |
| 49 | 3-Amino-8-chloroacridine | 0 | 0 | 0 | 0 | 0 | 0 | 4·1 | $<1$ |
| 50 | 5-Amino-1-chloroacridine | 6 | 5 | 4 | 3 | 2 | 20 | 8·0 | 83 |
| 51 | 5-Amino-2-chloroacridine | 7 | 6 | 5 | 5 | 3 | 26 | 8·7 | 96 |
| 52 | 5-Amino-3-chloroacridine | 7 | 6 | 5 | 4 | 4 | 26 | 8·5 | 94 |
| 53 | 5-Amino-4-chloroacridine | 6 | 6 | 4 | 4 | 2 | 22 | 8·1 | 86 |
| 54 | 2:8-Diamino-3:7-dichloro-acridine | 8 | 6 | 4 | 5 | 0 | 23 | 8·0 | 83 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 55 | 5-Amino-1-methoxyacridine | 7 | 6 | 4 | 3 | 2 | 22 | 9·7 | 100 |
| 56 | 5-Amino-2-methoxyacridine | 7 | 6 | 5 | 4 | 3 | 25 | 9·9 | 100 |
| 57 | 5-Amino-3-methoxyacridine | 7 | 7 | 5 | 4 | 2 | 25 | 9·4 | 99 |
| 58 | 5-Amino-4-methoxyacridine | 7 | 6 | 4 | 3 | 2 | 22 | 10·0 | 100 |
| 59 | 2:5-Diamino-7-ethoxy-acridine ('rivanol') . . | 6 | 7 | 4 | 3 | 0 | 20 | 11·2 | 100 |
| 60 | 5-Amino-2-chloro-7-methoxyacridine . . | 8 | 6 | 5 | 2 | 0 | 21 | 8·4 | 93 |
| 61 | 5-Amino-1-hydroxyacridine. | 5 | 5 | 3 | 3 | 3 | 19 | Z | 24 |
| 62 | 5-Amino-2-hydroxyacridine. | 2 | 4 | 0 | 0 | 0 | 6 | Z | 9 |
| 63 | 5-Amino-3-hydroxyacridine. | 4 | 6 | 2 | 0 | 0 | 12 | Z | 56 |
| 64 | 5-Amino-4-hydroxyacridine. | 3 | 3 | 1 | 0 | 0 | 7 | Z | 2 |
| 65 | 5-Amino-1-nitroacridine . | 6 | 7 | 4 | 4 | 3 | 24 | 7·6 | 67 |
| 66 | 5-Amino-2-nitroacridine . | 6 | 10 | 6 | 5 | 3 | 30 | 7·6 | 67 |
| 67 | 5-Amino-3-nitroacridine . | 7 | 6 | 5 | 4 | 2 | 24 | 7·7 | 72 |
| 68 | 5-Amino-4-nitroacridine . | 7 | 8 | 6 | 4 | 2 | 27 | 7·4 | 56 |
| 69 | 5-Amino-2-nitro-7-ethoxy-acridine . . . | 7 | 8 | 4 | 0 | 0 | 19 | 7·4 | 56 |
| 70 | 5-Amino-1-phenylacridine . | 5 | 5 | 3 | 2 | 0 | 15 | 9·0 | 98 |
| 71 | 5-Amino-3-phenylacridine . | 8 | 7 | 6 | 3 | 0 | 24 | 9·5 | 99 |
| 72 | 2-Amino-5-*p*-aminophenyl-acridine . . . | 6 | 4 | 3 | 1 | 0 | 14 | 8·0 | 83 |
| 73 | 2:8-Diamino-3:7-dimethyl-5-phenylacridine . . | 5 | 5 | 5 | 1 | 0 | 16 | 9·4 | 99 |
| 74 | 5-Amino-3-cyanoacridine . | 7 | 5 | 4 | 4 | 3 | 23 | 7·8 | 76 |
| 75 | 5-Methylaminoacridine . | 7 | 6 | 4 | 2 | 3 | 22 | 9·9 | 100 |
| 76 | 2-Dimethylaminoacridine . | 5 | 5 | 3 | 2 | 0 | 15 | 8·1 | 86 |

## APPENDIX I—*contd.*

| *No.* | *Substance* | *Organisms* | | | | | *Bacteriostatic Index (sum total of code numbers of inhibitory dilutions)* | *$pK_a$ in water at 37°* | *Per cent ionized (kation) at 37 °C. and pH 7·3* |
|---|---|---|---|---|---|---|---|---|---|
| | | Cl. welchii | Strept. pyogenes | Staph. aureus | B. coli | Proteus | | | |
| 77 | 2-Dimethylamino-7-amino-acridine . . . | 6 | 5 | 3 | 1 | 0 | 15 | 8·3 | 90 |
| 78 | 2:8-*bis*Dimethylamino-acridine . . . | 5 | 6 | 4 | 2 | 0 | 17 | 10·1 | 100 |
| 79 | 5-(β-Hydroxyethyl)-amino-acridine . . . | 5 | 5 | 2 | 2 | 1 | 15 | 9·1 | 98 |
| 80 | 10-Methylacridinium hydroxide . . . | 0 | 0 | 0 | 0 | 0 | 0 | (un-stable) | ? |
| 81 | 2-Amino-10-methylacridinium hydroxide . . . | 6 | 7 | 1 | 5 | 0 | 19 | 12 | 100 |
| 82 | 3-Amino-10-methyl-acridinium hydroxide . | 1 | 2 | 0 | 0 | 0 | 3 | (un-stable) | ? |
| 83 | 4-Amino-10-methyl-acridinium hydroxide . | 3 | 3 | 2 | 0 | 0 | 8 | (un-stable) | ? |
| 84 | 5-Amino-10-methyl-acridinium hydroxide . | 6 | 6 | 4 | 4 | 3 | 23 | 10·7 | 100 |
| 85 | 3-Amino-5:10-dimethyl-acridinium hydroxide . | 5 | 5 | 3 | 2 | 0 | 15 | 9·4 | 100 |
| 86 | 2:8-Diamino-10-methyl-acridinium hydroxide . | 7 | 8 | 3 | 3 | 1 | 22 | >12 | 100 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 87 | $\beta$-Amino-5-ethylacridine . | 6 | 6 | 2 | 3 | 2 | 19 | 8·9 | 98 |
| 88 | *p*-Amino-5-styrylacridine . | 0 | 0 | 0 | 0 | 0 | 0 | 5·5 | 2 |
| 89 | Acridine-3-sulphonic acid | 0 | 0 | 0 | 0 | 0 | 0 | Z* | <1 |
| 90 | Acridine-1-carboxylic acid | | | | | | | Z | <1 |
| 91 | Acridine-3-carboxylic acid | | | | | | | Z | <1 |
| 92 | Acridine-5-carboxylic acid | | | | | | | Z | <1 |
| 93 | Methyl ester of last named . | 0 | 0 | 0 | 0 | 0 | 0 | 3·5 | <1 |
| 94 | 2-Aminoacridine-7-sulphonic acid . . . . | 1 | 0 | 0 | 0 | 0 | 1 | Z | <1 |
| 95 | 2-Aminoacridine-7-sulphonamide . . . . | 4 | 2 | 0 | 2 | 0 | 8 | 6·9 | 29 |
| 96 | 2-Aminoacridine-7-carboxylic acid . . . . | 0 | 0 | 0 | 0 | 0 | 0 | Z | <1 |
| 97 | Methyl ester of last named . | 1 | 0 | 0 | 0 | 0 | 1 | 6·8 | 24 |
| 98 | 5-Aminoacridine-1-carboxylic acid . . . . | 0 | 0 | 0 | 0 | 0 | 0 | Z | <1 |
| 99 | 5-Aminoacridine-3-carboxylic acid . . . . | 0 | 0 | 0 | 0 | 0 | 0 | Z | <1 |
| 100 | Methyl ester of last named . | 7 | 6 | 5 | 2 | 0 | 20 | 8·3 | 90 |
| 101 | Amide of No. 99 . . | 3 | 5 | 2 | 3 | 2 | 15 | 8·5 | 94 |

From Albert, Rubbo, Goldacre, Davey and Stone (1945); Albert and Goldacre (1948). Many $pK_a$ values are revised, having been determined directly in water for the first time.

* Z=Zwitterion (see Albert and Goldacre, 1947).

# APPENDIX II

## CALCULATION OF PERCENTAGE IONIZED, GIVEN $pK_a$ AND pH

| $pK_a - pH$ | *if Anion* | *if Kation* |
|---|---|---|
| −6·0 | 99·99990 | 0·0000999 |
| −5·0 | 99·99900 | 0·0009999 |
| −4·0 | 99·9900 | 0·0099990 |
| −3·5 | 99·968 | 0·0316 |
| −3·4 | 99·960 | 0·0398 |
| −3·3 | 99·950 | 0·0501 |
| −3·2 | 99·937 | 0·0630 |
| −3·1 | 99·921 | 0·0794 |
| −3·0 | 99·90 | 0·09991 |
| −2·9 | 99·87 | 0·1257 |
| −2·8 | 99·84 | 0·1582 |
| −2·7 | 99·80 | 0·1991 |
| −2·6 | 99·75 | 0·2505 |
| −2·5 | 99·68 | 0·3152 |
| −2·4 | 99·60 | 0·3966 |
| −2·3 | 99·50 | 0·4987 |
| −2·2 | 99·37 | 0·6270 |
| −2·1 | 99·21 | 0·7879 |
| −2·0 | 99·01 | 0·990 |
| −1·9 | 98·76 | 1·243 |
| −1·8 | 98·44 | 1·560 |
| −1·7 | 98·04 | 1·956 |
| −1·6 | 97·55 | 2·450 |
| −1·5 | 96·93 | 3·07 |
| −1·4 | 96·17 | 3·83 |
| −1·3 | 95·23 | 4·77 |
| −1·2 | 94·07 | 5·93 |
| −1·1 | 92·64 | 7·36 |
| −1·0 | 90·91 | 9·09 |
| −0·9 | 88·81 | 11·19 |
| −0·8 | 86·30 | 13·70 |
| −0·7 | 83·37 | 16·63 |
| −0·6 | 79·93 | 20·07 |

## APPENDIX II—*contd.*

| $pK_a - pH$ | *if Anion* | *if Kation* |
|---|---|---|
| −0·5 | 75·97 | 24·03 |
| −0·4 | 71·53 | 28·47 |
| −0·3 | 66·61 | 33·39 |
| −0·2 | 61·32 | 38·68 |
| −0·1 | 55·73 | 44·27 |
| 0 | 50·00 | 50·00 |
| +0·1 | 44·27 | 55·73 |
| +0·2 | 38·68 | 61·32 |
| +0·3 | 33·39 | 66·61 |
| +0·4 | 28·47 | 71·53 |
| +0·5 | 24·03 | 75·97 |
| +0·6 | 20·07 | 79·93 |
| +0·7 | 16·63 | 83·37 |
| +0·8 | 13·70 | 86·30 |
| +0·9 | 11·19 | 88·81 |
| +1·0 | 9·09 | 90·91 |
| +1·1 | 7·36 | 92·64 |
| +1·2 | 5·93 | 94·07 |
| +1·3 | 4·77 | 95·23 |
| +1·4 | 3·83 | 96·17 |
| +1·5 | 3·07 | 96·93 |
| +1·6 | 2·450 | 97·55 |
| +1·7 | 1·956 | 98·04 |
| +1·8 | 1·560 | 98·44 |
| +1·9 | 1·243 | 98·76 |
| +2·0 | 0·990 | 99·01 |
| +2·1 | 0·7879 | 99·21 |
| +2·2 | 0·6270 | 99·37 |
| +2·3 | 0·4987 | 99·50 |
| +2·4 | 0·3966 | 99·60 |
| +2·5 | 0·3152 | 99·68 |
| +2·6 | 0·2505 | 99·75 |
| +2·7 | 0·1991 | 99·80 |
| +2·8 | 0·1582 | 99·84 |
| +2·9 | 0·1257 | 99·87 |
| +3·0 | 0·09991 | 99·90 |

APPENDIX II—*contd.*

| $pK_a - pH$ | *if Anion* | *if Kation* |
|---|---|---|
| +3·1 | 0·0794 | 99·921 |
| +3·2 | 0·0630 | 99·937 |
| +3·3 | 0·0501 | 99·950 |
| +3·4 | 0·0398 | 99·960 |
| +3·5 | 0·0316 | 99·968 |
| +4·0 | 0·0099990 | 99·9900 |
| +5·0 | 0·0009999 | 99·99900 |
| +6·0 | 0·0000999 | 99·99990 |

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